The function of Themis2 in B cells

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Declaration of originality

I, Harald Hartweger, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Thymocyte-expressed molecule involved in selection 2 (*Themis2*) is the second member of the Themis family. Recently, the first member of the Themis family, Themis, has been reported to be part of the TCR signalling cascade and its deletion severely affects thymocyte progression from the double positive to the single positive stage. All family members share similar domains and high sequence similarity and show tissue specific expression with *Themis2* being expressed in B lymphocytes, macrophages and dendritic cells. THEMIS2 associates with BCR signalling molecules such as GRB2, VAV or LYN and is phosphorylated in response to BCR stimulation. For these reasons I hypothesised that *Themis2* might have an important role in B cell development or activation.

I show that *Themis2* is expressed throughout the B cell lineage and exclude redundant expression of other Themis family members. After B cell activation *Themis2* expression is downregulated. Analysis of a newly created *Themis2*-deficient mouse strain showed that B cell development proceeds normally in the absence of THEMIS2. Experiments on *in vitro* cultured *Themis2*-deficient primary B cells demonstrated that proliferation and survival, BCR internalisation and antigen presentation as well as expression of activation markers and cytokines were unaffected. RNA sequencing revealed only minor changes in transcription in follicular B cells, even after activation. Similarly, antibody levels to i*n vivo* immunisation with T-dependent or T-independent antigens or challenge

with influenza virus did not suggest that *Themis2* is required for antibody responses either. Reactions to a model of acute allergic airway inflammation showed only marginally reduced cell numbers in the bronchoalveolar lavage fluid yet all other markers of inflammation were all normal. In conclusion, I found that *Themis2* is not required for B cell development, activation or antibody responses. Further studies will be required to define the role of *Themis2* in the immune system.

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Abbreviations

A	Adenosine
AFC	Antibody secreting cell
AID	Activation-induced cytidine deaminase
AIRE	Autoimmune regulator
AKT	Thymoma viral proto-oncogene 1
AP-1	Activator protein-1
APC	Antigen-presenting cell
APE1	Apurinic/apyrimidinic endonuclease 1
APRIL	A proliferation inducing ligand
APS	Ammonium persulfate
ARG1	Arginase 1
ATF-2	Activating transcription factor 2
BACH2	BTB and CNC homology 1, basic leucine-zipper transcription factor 2
BAFF	B cell-activating factor
BAFFR	B cell-activating factor receptor
BASH	B cell adaptor containing SH2 domain
BCL2L11	B cell leukemia/lymphoma 2-like 11
BCL6	B cell leukemia/lymphoma
BCMA	B cell maturation antigen
BCR	B cell receptor
β-Gal	Beta-Galactosidase
BIM	BCL2-interacting mediator of cell death
BLIMP1	B lymphocyte-induced maturation protein 1
BLNK	B cell linker
BMDM	Bone marrow derived macrophage
Bregs	Regulatory B cells
BTK	Bruton's tyrosine kinase
C gene	Constant region gene segment
c-JUN	Jun proto-oncogene

c-MYC	Myelocytomatosis oncogene
CABIT	Cysteine-containing, all-β in Themis
CBLB	Casitas-B-lineage lymphoma protein-B
CD	Cluster of differentiation
CD40L	Cluster of differentiation 40 ligand
CDR	Complementarity determining region
CDS	Coding sequence
CLP	Common lymphoid progenitor
CLR	C-type lectin receptors
CN	Calcineurin phosphatase
COX2	Cyclo-oxygenase 2
CRAC	Ca^{2+} release-activated Ca^{2+} channel
cSMAC	Central supramolecular activation cluster
CSR	Class switch recombination
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCR5	Chemokine (C-X-C motif) receptor 5
D gene	Diversity region gene segment
DAG	Diacylglycerol
DAMP	Danger-associated molecular patterns
DC	Dendritic cell
dC	Deoxycytidine
dG	Deoxyguanosine
DL1	Delta-like 1
DMSO	Dimethyl sulfoxide
DN	Double negative thymocyte
DNA	Deoxyribonucleic acid
DP	Double positive thymocyte
DSB	Double strand break
dsRNA	Double-stranded ribonucleic acid
dU	Deoxyuridine
EAE	Experimental autoimmune encephalomyelitis

EBF1	Early B cell factor 1
EGF	Epidermal growth factor
ELK1	ETS domain-containing protein
EN2 SA	EN2 splice acceptor site
ERE	Oestrogen response element
ERK	Extracellular signal-regulated kinase
ERα	Oestrogen receptor alpha
eTAC	Extrathymic Aire-expressing cells
FDG	Fluorescein di-(β-D-galactopyranoside)
FDR	False discovery rate
Fizz1	Found in inflammatory zone 1
FLT3	FMS-like tyrosine kinase 3
FOB	Follicular B cells
FOXP3	Forkhead box P3
GAB1	Growth factor receptor bound protein 2-associated binder
GADS	Grb2-related adaptor downstream of Shc
γ_c	IL-2 receptor common gamma chain
GAREM	Grb2 associated, regulator of MAPK1
GAREML	Grb2 associated, regulator of MAPK1-like
GATA3	GATA binding protein 3
GC	Germinal centre
GCB	Germinal centre B cell
GRB2	Growth factor receptor bound protein 2
HBSS	Hank's balanced salt solution
HER2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2
HSC	Haematopoetic stem cell
I-exon	Intervening exon
IBD	Inflammatory bowel disease
ICAM1	Intercellular adhesion molecule 1
ICB-1	Induced by contact to basement membrane-1
ICOS	Inducible T cell co-stimulator
IFN	Interferon

Ig	Immunoglobulin
IKK	Inhibitor of κ B kinase
IL	Interleukin
IL-4R	Interleukin-4 receptor
IL-7R	Interleukin-7 receptor
Iono	Ionomycin
IP_3	Inositol-3,4,5-phosphate
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
IRS	Insulin receptor substrate
ITAM	Immunoreceptor tyrosine-based activation motif
iTreg	Inducible regulatory T cell
IκB	Inhibitor of κB
J gene	Joining region gene segment
JAK	Janus kinase
JNK	C-Jun NH2-terminal kinase
KLF2	Krüppel-like factor 2
KO	Knock-out
LAB	Linker for activation of B cells
LAT	Linker for activation of T cells
LFA1	Lymphocyte function-associated antigen 1
LMPP	Lymphoid-primed multipotent progenitors
LN	Lymph node
LP	Lamina propria
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTα	Lymphotoxin alpha
M-CSF	Macrophage-colony-stimulating factor
MAML1	Mastermind-like 1
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAPK	Mitogen-activated protein kinases
MEKK1	Mitogen-activated protein kinase kinase kinase 1

MHC	Major histocompatibility complex
mLN	Mesenteric lymph nodes
MPP	Multipotent progenitors
MTA3	Metastasis-associated 1 family, member 3
μHC	Mu heavy chain
MYD88	Myeloid differentiation primary response gene 88
MZ	Marginal zone
MZB	Marginal zone B cell
MZP	Marginal zone precursor cell
N_{BH}	B cell helper neutrophil
Neo^R	Neomycin resistance
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κ-light chain-enhancer of activated B cells
NIK	NF-κB-inducing kinase
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NKT_{FH} cells	Follicular helper natural killer T cell
NLR	NOD-like receptors
NLS	Nuclear localisation sequence
NOD	Nucleotide-binding oligomerisation domain
NP	4-Hydroxy-3-nitrophenylacetyl
NTAL	Non-T cell activation linker
nTreg	Natural regulatory T cell
Omp	Outer membrane protein
pA	Polyadenylation signal
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen-associated molecular patterns
PAX5	Paired box gene 5
PB	Plasmablast
PC	Plasma cell
PD-1	Programmed cell death 1
PD-L2	Programmed cell death ligand 2

PDK1	Phosphoinositide-dependent kinase 1
PEC	Peritoneal exudate cells
PETG	2-Phenylethyl β-D-thiogalactoside
PGN	Peptidoglycan
PI3K	Phosphoinositide-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PLCγ2	Phospholipase C gamma 2
pLN	Peripheral lymph nodes
PMA	Phorbol-12-myristate-13-acetate
pMHC	Peptide-major histocompatibility complex molecules
PNA	Peanut agglutinin
PP	Peyer's patches
PRR	Pattern recognition receptor
PRS	Proline-rich SH3-binding site
PTC	Premature termination codon
pTreg	Peripheral regulatory T cell
PU.1	Purine box factor 1
RAC	RAS-related C3 botulinum substrate
RAF1	V-Raf-leukemia viral oncogene 1
RAG	Recombination activating gene
RANK	Receptor activator of nuclear factor-κB
RAR	Retinoic acid receptor
RAS	Resistance to audiogenic seizures
RASGRP	Resistance to audiogenic seizures guanyl releasing protein
RBP-Jκ	Recombination signal binding protein for immunoglobulin kappa J region
RIG-I	Retinoic acid-inducible gene
RIP1	Receptor-interacting serine/threonine-protein kinase 1
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference

RORγt	Retinoic acid receptor-related orphan receptor gamma t
RSS	Recombination signal sequences
S region	Switch region
S1PR	Sphingosine-1-phosphate receptor
SAM	Sterile α-motif
SAP	Signalling lymphocyte activation molecule–associated protein
SH2	Src homology 2
SH3	Src homology 3
SHM	Somatic hypermutation
SHP	SH2 domain-containing protein tyrosine phosphatase
sIg	Surface immunoglobulin
siRNA	Small interfering ribonucleic acid
SLAM	Signalling lymphocyte activation molecule
SLP-65	SH2 domain–containing leukocyte protein of 65 kD
SLP-76	SH2 domain–containing leukocyte protein of 76 kD
SMUG1	Single-strand selective monofunctional uracil DNA glycosylase
SNP	Single nucleotide polymorphism
SOS	Son of sevenless homologue
SP	Single positive thymocyte
ssRNA	Single stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
STIM	Stromal interaction molecules
SYK	Spleen tyrosine kinase
T	Thymidine
T-BET	T-box expressed in T cells
T1/2/3	Transitional stage 1/2/3
T2A	T2A self-cleaving oligopeptide
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TAK1	TGFβ-activated kinase
TANK	TRAF family member-associated NFκB activator

TBK1	TANK-binding kinase 1
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TEMED	Tetramethylethylenediamine
Tfh	T follicular helper
Tfr	Follicular regulatory T cell
TGFβ	Transforming growth factor beta
Th	T helper
THEMIS	Thymocyte-expressed molecule involved in selection
TICAM2	Toll-like receptor adaptor molecule 2
TIR domain	Toll/IL-1 receptor homology domain
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNFR	Tumour necrosis factor-receptor
TNFα	Tumour necrosis factor alpha
TNP	Trinitrophenyl
TRAF	TNF-associated factor
Treg	Regulatory T cell
TRIF	TIR-domain-containing adaptor protein inducing IFNβ
tTreg	Thymic regulatory T cell
TWEAK	TNF-related weak inducer of apoptosis
Ub	Ubiquitin
UNG	Uracil DNA glycosylase
UTR	Untranslated region
V gene	Variable region gene segment
VCAM1	Vascular cell adhesion molecule 1
VEGF-A	Vascular endothelial growth factor A
VLR	Variable lymphocyte receptor
WASP	Wiscott-Aldrich syndrome protein
WT	Wild type
Xid	X-linked immunodeficiency

XLA	X-linked agammaglobulinaemia
Ym1	Chitinase-like 3
ZAP70	Zeta-chain associated protein kinase 70

1 Introduction

1.1 The immune system

The immune system of mammals can be divided into the innate and adaptive immune system. Both systems are primarily designed to prevent the host from succumbing to infection but the immune system also has surveillance functions in preventing cancer and detecting sterile damage leading to wound healing. The innate immune system consists of barriers such as the skin and mucosal surfaces and of innate immune cells such as macrophages, dendritic cells and neutrophils (Iwasaki and Medzhitov, 2010; Medzhitov, 2007; Murray and Wynn, 2011). The adaptive immune system, based on the clonal selection theory proposed by Sir MacFarlane Burnet, is composed of lymphocytes, which develop antigen specific receptors (Sir Frank MacFarlane, 1959). Historically, it can be subdivided into humoral and cellular immunity based on B and T cell lineages respectively. Humoral immunity refers to the production of antibodies by B cells whereas cellular immunity refers to cytotoxic T lymphocytes, which directly attack and destroy target cells. However, cellular immunity also refers to cells of the innate immune system, in particular phagocytes, which similarly to cytotoxic T cells, eliminate infected cells. Their increased phagocytic capacity is increased by T helper (Th) cells, another cell type considered part of cellular immunity. As such, a strict division would leave out crucial interactions between cells of both cellular and humoral immunity, which is why this division is indistinct (Silverstein, 2003).

Equally, the innate and the adaptive immune system are also connected in orchestrating the immune response as a whole (Schenten and Medzhitov, 2011; Shanker, 2010).

1.1.1 Innate immunity

The innate immune system is considered the first barrier to invading pathogens. Its roots are ancient and similar mechanisms are found in the most primitive metazoans such as sponges (Hirano et al., 2011). Cells of the innate immune system use pattern recognition receptors (PRRs) to detect conserved molecules of pathogens or signs of cellular damage, which are called pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) respectively (Kawai and Akira, 2009; Petrilli et al., 2007). The most prominent class of these receptors are Toll-like receptors (TLRs) which are horseshoe-shaped transmembrane receptors located at either the cell surface or within endosomes. They detect a range of PAMPs using leucine-rich repeat (LRR) sequences including bacterial cell wall components such as lipopolysaccharide (LPS) or peptidoglycan (PGN) or viral ribonucleic acids (RNAs) such as double-stranded RNA (dsRNA), single stranded RNA (ssRNA) and CpG deoxyribonucleic acid (CpG DNA). Other classes of PRRs are retinoic acid-inducible gene (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs) (Geijtenbeek and Gringhuis, 2009; Kawai and Akira, 2009). RLRs and NLRs are located in the cytoplasm and

generally detect foreign RNA species whereas CLRs are membrane proteins mostly recognizing fungal and bacterial PAMPs (Kawai and Akira, 2011). Engagement of these receptors leads to activation of signalling pathways, which ensure tailored immune responses by both the innate and the adaptive immune system in line with the theory initially proposed by Charles Janeway (Janeway, 1989).

1.1.2 Adaptive immunity

Vertebrates have developed an adaptive immune system in addition to the innate immune system. Two divergent adaptive immune systems using recombinatorial receptors have arisen during evolution. Jawless vertebrates have an immune system based on variable lymphocyte receptors (VLRs), which use LRR motifs for antigen binding. Jawed vertebrates in contrast use an immunoglobulin (Ig)-domain based system to produce T cell receptors (TCR) and B cell receptors (BCR). Both systems have in common that they are subdivided into two separate lymphocyte branches and that activation-induced cytidine deaminase (AID) is used for receptor diversification (Hirano et al., 2011). The recombinatorial nature of such a systems enables the adaptive immune system to construct diverse, anticipatory receptors, specific for possible, future antigens. Thus an immune response against specific antigens can be mounted by the adaptive immune system (Bonilla and Oettgen, 2010; Hirano et al., 2011).

1.1.2.1 Cellular immunity

Cellular immunity is mostly referred to immunity mediated by cluster of differentiation (CD) 8-positive ($CD8^+$) cytotoxic T lymphocytes (CTLs) and $CD4^+$ T helper (Th) cells. These T cells develop in the thymus. T-lymphoid progenitors from the bone marrow seed the thymic parenchyma from vessels in the corticomedullary junction of the thymus and travel during their development though the cortex towards the capsule and then back into the medulla to then emigrate to peripheral lymphoid organs though blood vessels as recent thymic emigrants (RTEs) (Takahama, 2006). $CD4^-$ $CD8^-$ double negative (DN) thymocytes are the earliest T cell progenitors in the thymus and are divided into 4 sequential stages, DN1 to DN4, depending on the expression of CD44 and CD25. Lineage commitment is not finished until the DN2 stage. TCRβ or TCR$\gamma\delta$ rearrangement occurs at the DN3 stage and cells are tested for successful recombination at the following β-selection checkpoint. Expression of a pre-TCR consisting of the rearranged TCRβ chain, pre-Tα and CD3 chains lets the cells progress into the DN4 stage and then the $CD4^+$ $CD8^+$ double positive cell (DP) stage. Cells that diverge into the TCR$\gamma\delta$ lineage recombine both the γ and δ chain and the rearrangement is checked together (Carpenter and Bosselut, 2010).

DP cells rearrange their TCRα chains and are subject to positive selection for a functional TCR$\alpha\beta$ complex that recognises peptide-major histocompatibility complex molecules (pMHC). Negative selection ensures that this complex does not recognise autoantigens and in cells with such

autoreactive TCRs apoptosis is induced. Lack of signalling due to non-productive TCR rearrangements leads to death by neglect of DPs whereas excessive signalling through the TCR induced by autoantigens leads to activation-induced cell death (Carpenter and Bosselut, 2010; Fu et al., 2014). The signalling strength through the TCR is thought to determine CD4 versus CD8 lineage choice. If a αβTCR interacts with MHC class II as opposed to MHC class I, it receives a stronger signal as the co-receptor for MHC class II is CD4. CD4 has stronger interactions with the downstream signalling molecule LCK than the MHC class I-restricted CD8 co-receptor, thus CD4 lineage decision is made by stronger TCR signalling and the CD8 lineage is preferred for lower-strength TCR signals (He et al., 2010). In addition, $CD4^+$ thymocytes with particularly strong affinity for self-antigen and thus strong TCR signalling are diverted into the regulatory T cell lineage (Carpenter and Bosselut, 2010; Josefowicz et al., 2012). Termination of either CD4 or CD8 expression marks the transition of the DP thymocytes to the single positive thymocytes (SP), which emigrate from the thymus into the periphery as RTEs (Carpenter and Bosselut, 2010). RTEs mature in the periphery to become mature T cells which can exert T cell effector functions. The maturation process in RTEs is thought to reset downstream signalling processes so that cells respond with T cell activation and effector mechanisms rather than with activation-induced cell death (Fink, 2013).

The principal effector function of $CD8^+$ CTLs is direct killing of target cells infected with intracellular pathogens such as viruses or intracellularly

dividing bacteria. Th cells are regulators of other immune cells and enable effective killing of phagocytosed pathogens by for example macrophages as well as helping B cells recognizing a foreign antigen to get activated and differentiate into antibody secreting plasma cells and form germinal centre B cells (GCB) (Bonilla and Oettgen, 2010; Shanker, 2010).

Th cells are classified into different subsets, which develop upon activation of the cell. Naïve Th cells are termed Th0 before activation and give rise to T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), regulatory T cells (Treg), T follicular helper cells (Tfh) or the recently described T helper 9 (Th9) subsets depending on the antigen-presenting cell (APC) and the cytokine milieu.

Th1 cells are induced by interleukin (IL)-12, characterised by having the lineage transcription factor T-box expressed in T cells (T-BET) and producing mainly interferon (IFN)γ and IL-2. Th1 cells generally promote cell-mediated responses by activating mononuclear phagocytes, natural killer (NK) cells and CTLs for effective killing of intracellular pathogens.

Th2 cells are induced by IL-4, have GATA binding protein 3 (GATA3) as their lineage transcription factor and produce IL-4, IL-5 and IL-13 and have functions in enhancing antibody responses, in fighting helminth infections and in hypersensitivity (Bonilla and Oettgen, 2010).

Th17 cells are induced by IL-6 and transforming growth factor β (TFGβ) (Bettelli et al., 2006; Veldhoen et al., 2006), characterised by retinoic acid receptor (RAR)-related orphan receptor γ t (RORγt) as their lineage transcription factor (Ivanov et al., 2006) and produce mainly IL-17A and IL-

17F which are potent proinflammatory cytokines driving IL-6 and tumour necrosis factor α (TNFα) expression and granulocyte recruitment (Bonilla and Oettgen, 2010; Harrington et al., 2005; Park et al., 2005). Th17 cells have been implicated in several autoimmune diseases including arthritis, multiple sclerosis and systemic lupus erythematosus and inflammatory bowel disease (Bonilla and Oettgen, 2010; Langrish et al., 2005; Marwaha et al., 2012).

Th9 cells have been described as IL-9 producing cells, which are reprogrammed from Th2 cells using transforming growth factor β (TGFβ) and IL-4 (Dardalhon et al., 2008; Veldhoen et al., 2008). These cells have been implicated in ulcerative colitis (Gerlach et al., 2014).

In vivo Tregs can either be generated in the thymus by high-affinity autoantigen interactions, termed thymic or natural Tregs (tTregs or nTregs), or they can be induced in the periphery by subimmunogenic antigen presentation as peripheral Tregs (pTregs) (Curotto de Lafaille and Lafaille, 2009). *In vitro* differentiated Tregs are referred to as induced Tregs (iTregs) (Povoleri et al., 2013). Differentiation into Tregs is induced by TGFβ and IL-2 (Josefowicz et al., 2012). They are characterised by the expression of the transcription factor forkhead box P3 (FOXP3) and they suppress proinflammatory processes through a variety of secreted and surface molecules including IL-10, TGFβ, cytotoxic T-lymphocyte-associated protein 4 (CTLA4), CD39 and CD73 (Chaudhry and Rudensky, 2013; Fontenot et al., 2003; Kim and Rudensky, 2006).

Tfh cells are characterised by the expression of chemokine (C-X-C motif) receptor 5 (CXCR5) enabling them to migrate into the B cell follicle to provide help to B cells in the germinal centre reaction and the CD28 family member programmed cell death 1 (PD-1) and inducible T cell co-stimulator (ICOS). They express the lineage transcription factor B cell leukaemia/lymphoma 6 (BCL6) and their hallmark cytokine IL-21 and express CD40 ligand (CD40L) which is necessary to select B cell clones with higher affinity antibodies after somatic hypermutation in the germinal centre (Johnston et al., 2009; Nurieva et al., 2009; Rolf et al., 2010; Victora and Nussenzweig, 2012; Vogelzang et al., 2008).

1.1.2.2 Humoral immunity

Humoral Immunity is defined as immunity mediated by antibodies found in the extracellular body fluids (or humours) such as blood and lymph. Antibodies are produced by plasma cells, which are a terminally differentiated B cell subset. To reach this end stage of differentiation and produce antibodies, B cells not only need antigen-induced signalling by the BCR but also signalling through other receptors, which are often activated by ligands from other cells such as dendritic cells and T cells. This is crucial for B cells to follow appropriate differentiation pathways during development, perform class switch recombination (CSR) to produce the adequate antibody isotype and to perform somatic hypermutation (SHM) to increase antibody affinity (Bonilla and Oettgen, 2010; Clark et al., 2014; Oracki et al., 2010).

1.2 B cell development

In adult mammals B cells develop in the bone marrow (Kurosaki et al., 2010; Pieper et al., 2013). After their development in the bone marrow they travel to peripheral organs such as the spleen and lymph nodes and progress into the naïve, mature B cell pool where they survey the environment for foreign material using the BCR. If B cells encounter cognate BCR ligands they become activated and differentiate further into germinal centre B cells, memory B cells and plasma cells. An overview of the standard model of B cell development in given in Figure 1.

1.2.1 B cell development in the bone marrow

1.2.1.1 Development of B lineage precursors

B cell development is preceded by a first wave of haematopoiesis in the mouse foetus at day E7.5 in the yolk sac whilst blood circulation is established (Moore and Metcalf, 1970). A second wave gives rise to the first lymphoid cells among other cell types derived from haematopoietic progenitor cells originating from the aorta-gonad-mesonephros area of the developing foetus (Sánchez et al., 1996). The first B-lymphoid cells and myeloid cells develop in one wave from the foetal liver on day E12.5 (Strasser, 1989) and from E13.5 onwards, B cell development proceeds continuously from pluripotent haematopoietic stem cells (HSCs) in the bone marrow as it does later on in the adult life (Arai et al., 2009; Tsuneto et al., 2014).

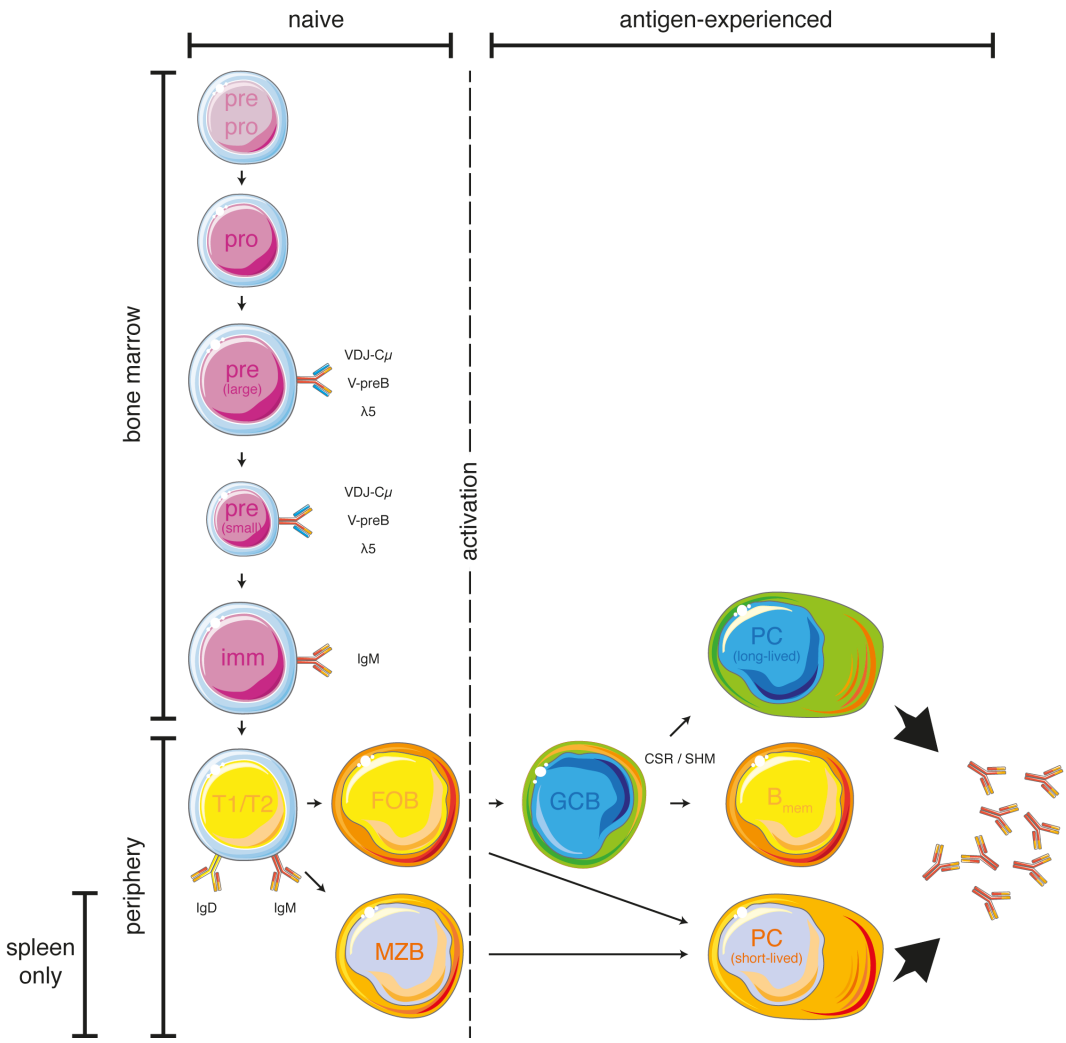

Figure 1: Canonical view of B cell development.

CLP-derived prepro-B cells develop into pro-B cells, which start to rearrange their immunoglobulin heavy chain. Upon successful recombination and expression of a rearranged VDJ-Cμ heavy chain and co-expression of V-preB and λ5, the pre-BCR is formed and cells become large blasting pre-B cells to expand successfully rearranged clones. After blasting, cells start rearranging their κ light chain locus or, upon unsuccessful recombination of the κ locus, initiate rearrangement of the λ light chain locus. Successful recombination on either of these loci produces immature B cells expressing the complete BCR in the form of IgM. These cells leave the bone marrow and migrate to the periphery where they start to co-express IgD on their surface through differential

splicing and transit through the T1 and T2 transitional stages to become mature follicular B cells. In the spleen, mature marginal zone B cells can also develop from T2 B cells. Upon activation by cognate antigen, follicular B cells can either become a short lived plasma cell (PC) or go into the germinal centre reaction to produce other antibody isotypes by class switch recombination (CSR) and increase antibody affinity by somatic hyper mutation (SHM). The latter, germinal centre B cells (GCB) then develop either into affinity–matured memory B cells (B_{mem}) or even more highly affinity-matured, long-lived antibody-producing PCs, which migrate into a survival niche in the bone marrow and sustain humoral immunity. Marginal zone B cells can also form memory but generally are fast responders to produce short-lived PCs. More details can be found in the text.

When differentiating, HSCs lose self-renewing potential and become multipotent progenitors (MPPs), which keep on losing the capacity to differentiate into different haematopoietic lineages and become more and more committed towards one lineage (Bryder and Sigvardsson, 2010). The commitment towards the lymphoid lineage is induced by the transcription factor IKAROS in these early progenitor cells (Georgopoulos et al., 1992; Ng et al., 2009). The first commitment to the lymphoid lineage is marked by low expression of recombination activating gene 1 (RAG1) (Igarashi et al., 2002). MPPs then develop into lymphoid-primed multipotent progenitors (LMPPs), which can still produce granulocytes and macrophages but not erythrocytes or megakaryocytes (Adolfsson et al., 2005). LMPPs are induced by the action of the transcription factor E2A (Dias et al., 2008). These then give rise to common lymphoid progenitors (CLPs) in which myeloid potential is strongly reduced and which can almost exclusively produce lymphoid lineages (defined as NK, B and T cells) (Kondo et al., 1997). CLPs express the receptors FMS-like tyrosine kinase 3 (FLT3) and the IL-7 receptor (IL-7R) which are both vital for development of B cells at this stage but not earlier on in commitment towards the B cell lineage (Sitnicka et al., 2003; Sitnicka et al., 2002). Expression of IL-7R is controlled by the transcription factor purine box factor 1 (PU.1) (DeKoter et al., 2002), however higher levels of PU.1 inhibit B cell development and favour development of macrophages (DeKoter, 2000). The IL-7R regulates B cell lineage commitment through the regulation of the expression of the transcription factor early B cell factor 1

(EBF1) (Dias et al., 2005). It has been suggested that EBF1 is the critical factor for this commitment to the B lymphoid lineage in CLPs (Zandi et al., 2008). CLPs develop into $B220^+$ $CD19^-$ pre-pro B cells, which already show strong commitment to the B cell lineage (Rumfelt et al., 2006) (Mansson et al., 2010). However, some of these cells can still seed the thymus and become T cells (Martin et al., 2003). In fact, CLPs can commit strongly to the B cell lineage before they progress into the $B220^+$ $CD19^-$ pre-pro B cell stage indicated by a λ5-reporter mouse. However, when reaching the $B220^+$ $CD19^-$ pre-pro B cell stage, cells lack myeloid potential and show little commitment to the T cell lineage (Mansson et al., 2008). Since RAG1 expression is already turned on, cells begin rearranging their IgH locus. As not all cells are committed to the B cell lineage, D to J rearrangements at the IgH locus can also be found even in T cells (Born et al., 1988). E2A, EBF1 and PU.1 together control the expression of the transcription factor paired box gene 5 (PAX5) (O'Riordan and Grosschedl, 1999). PAX5 is a crucial transcription factor for commitment to the B cell lineage at this stage (Nutt et al., 1999) and is essential for maintaining B cell lineage commitment in mature B cells as well (Cobaleda et al., 2007a). It has thus been called the guardian of B cell identity and function (Cobaleda et al., 2007b). PAX5 is hardly detectable in CLPs or pre-pro B cells and its expression coincides with full commitment to the B cell lineage (Fuxa and Busslinger, 2007). The $CD19^+$ pro-B cell stage marks the stage of full commitment to the B cell lineage as opposed to earlier progenitors, which can still produce other lineages (Rumfelt et al., 2006).

1.2.1.2 Development of the B lineage

Early developmental B cell stages have been split up using two sets of surface markers to characterise the cells by flow cytometry. The two commonly used nomenclatures ranging from pre-pro B cells to mature B cells are the Philadelphia nomenclature (Hardy et al., 1991; Hardy and Hayakawa, 2001; Li et al., 1996) which is sometimes split into fractions A to F instead of naming each developmental stage and the Basel nomenclature (Osmond et al., 1998). Here, the Philadelphia nomenclature is used throughout.

Pro B cells rearrange their variable (V), diversity (D) and joining (J) gene segments of the IgH locus with D_H - J_H rearrangements preceding V_H - DJ_H rearrangements (Chowdhury and Sen, 2001). This process is dependent on IL-7, PAX5, the transcription factor Yin Yang 1 (YY1) and high levels of RAG1 and RAG2 (Liu et al., 2007; Oettinger et al., 1990; Xu et al., 2008).

Successful rearrangement of the VDJ_H locus results in expression of the pre-BCR composed of the μ heavy chain (μHC) and the two surrogate light chains Vpre-B and λ5 which are expressed together on the surface (Kudo and Melchers, 1987; Mårtensson et al., 2010; Sakaguchi and Melchers, 1986; Tsubata and Reth, 1990). Cells expressing the pre-BCR are termed pre-B cells. Signalling by the pre-BCR and the IL-7R leads to proliferation of large pre-B cells via phosphoinositide-3 kinase (PI3K) and extracellular signal-regulated kinase (ERK) signalling pathways (Fleming and Paige, 2001). The ligand for the pre-BCR or whether a ligand is required at all is currently unknown and several hypotheses on how the signalling is

initiated exist (Mårtensson et al., 2010). A second function of the pre-BCR checkpoint is to stop recombination of second IgH allele by allelic exclusion and prevent expression of more than one receptor in a single B cell. This has been shown to be dependent on pre-BCR surface expression (Boekel et al., 1998) and on downstream signalling molecules such as spleen tyrosine kinase (SYK) and zeta-chain associated protein kinase 70 (ZAP70) among others (Schweighoffer et al., 2003).

After proliferation, pre-B cells enter the G1 phase of the cell cycle, upregulate the *Rag* genes again and start recombination of the IgL chain to complete the production of a functional BCR (Kurosaki et al., 2010). There are two light chain loci, *Igκ* and *Igλ*. Interestingly, in mice κ is the preferred locus for the first attempt of creating a functional light chain. Twenty times more κ light chains are found in mice than λ light chains whereas in humans the ratio is roughly 1:1. The reasons for this species difference and the κ preference in mice is unknown but it has been shown to be independent of antigenic selection as the ratio of κ to λ is the same in immature as in mature B cells (McGuire and Vitetta, 1981). The activation of recombination is achieved by enhanced accessibility of the locus, which is exploited by the transcription factors interferon regulatory factor 4 (IRF4) and PU.1 (McDevit et al., 2005). Both access of IRF4 and reduced signalling by IL-7 mediate the activation of light chain recombination potentially by upregulation of CXCR4, which mediates migration away from IL-7 producing stromal cells (Johnson et al., 2008). Allelic exclusion of other *IgL* alleles is achieved through differential

methylation (Mostoslavsky et al., 1998) and a regulatory region between V_H and D genes termed intergenic control region 1 containing CTCF looping/insulator factor-binding elements (Guo et al., 2011).

The complete BCR consists of 2 heavy chains, 2 light chains and the functional subunits Igα and Igβ (also known as CD79A and CD79B respectively) and B cells expressing only the μ heavy chain but not the δ heavy chain are termed immature B cells (Kurosaki et al., 2010).

BCRs are highly diverse in their paratopes, which are the antigen binding regions. The joining of the different *VDJ* genes is one of the mechanisms to create a diverse BCR repertoire against foreign antigens (Tonegawa, 1983). The IgH locus in C57BL/6 mice extends over ~ 3 Mb and consists of 110 functional *V* genes and 85 *V* pseudogenes, 10 *D* genes, 4 *J* genes and 8 constant region (C) genes (Johnston et al., 2006). However, differences in the exact number of genes of the locus are found between mouse strains (Ye, 2004). Both heavy and light chain loci together allow more than 10^{12} combinations to be made in the mouse (Mårtensson et al., 2010). In comparison, the human *IgH* locus contains 123 V_H genes of which 79 are pseudogenes (Matsuda et al., 1998) indicating that these loci are quite different between species and that the number of genes is not the only mechanism to diversify the receptor. Complementarity determining region 3 (CDR3) of the IgH (and the TCRβ chain) has been shown to be the most important part of the BCR (and TCR) for determination of its specificity (Xu and Davis, 2000). CDR3 is encoded by the recombined V_H to D_H junction, the whole D_H gene segment as well as

the D_H *to* J_H junction (Davies and Chacko, 1993; Zhao and Lu, 2010). These junctions are particularly diverse as the enzyme terminal deoxynucleotidyl transferase (TdT) adds so-called N nucleotides at the recombination break points by template-independent synthesis. Thus, TdT is a major contributor to antibody diversity (Gilfillan et al., 1993). Some of the created combinations will recognise foreign antigen, others however might recognise self-antigen or will be out of frame or fold wrongly.

1.2.2 Selection and tolerance in B cell development

Consequently, cells at the immature B cell stage are subject to positive and negative selection to test whether the newly formed BCR is functional. A functional BCR gives a signal for positive selection, but if reacting too strongly, i.e. with self-antigen, leads to activation-induced cell death, also termed deletion. As a rescue mechanism, the BCR can be changed by a process called receptor editing in which usually the light chains are again recombined to allow testing of a new BCR specificity (Luning Prak et al., 2011) Alternatively, cells can go into anergy or clonal ignorance (Pillai et al., 2011). Anergic B cells still migrate to the periphery but do not respond to antigenic stimulation (Yarkoni et al., 2010) whereas ignorant B cells, which only have weakly self-reactive BCRs, are stopped from being activated by having a high activation threshold.

Receptor editing is the most common outcome of creating a non-functional receptor during B cell development (Hippen et al., 2005). This means another round of recombination occurs leading to use of different *V*, *D* or *J*

genes. It most commonly happens at the light chain loci as in the heavy chain usually only DJ_H leapfrogging occurs as otherwise the 12/23 rule is broken. The 12/23 rule states that recombination occurs preferentially between recombination signal sequences (RSS) with different spacer lengths. RSS are signal sequences for recombination and consist of a conserved heptamer sequence and a nonamer sequence separated by a spacer. In the mouse heavy chain locus V_H genes and J_H genes have 23 nucleotide long RSS spacers and D_H genes have 12 nucleotide long RSS spacers. Thus D_H genes recombine with both V_H and J_H genes but V_H genes do not recombine with J_H genes (Sakano et al., 1981). However, this rule is only a preference and recombinations breaking the rule have been reported (Vinocur et al., 2009).

DJ_H leapfrogging denotes an upstream D_H gene segment recombining with a downstream J_H gene segment thus deleting the already recombined DJ_H segment in between. This is only possible if the V_H genes have not been recombined and is thus found mainly early in development. (Luning Prak et al., 2011). However, for receptor editing at the immature B cell stage V_H to V_H recombination can occur using a cryptic RSS replacing only part of the originally recombined V_H gene, leaving the 3' part of the original V_H in place and thus usually leaving the CDR3 sequence untouched (Luning Prak et al., 2011). It also has been recently reported that receptor editing of $V\kappa$ genes can be found not only in the bone marrow of mice but also in the lamina propria (LP) from around day 18 to 28 after birth suggesting

that gut microbiota can influence the mature B cell repertoire (Wesemann et al., 2013).

If receptor editing fails, immature B cells with self-reactive BCRs can go into anergy. Anergy is based on the theory that lymphocyte activation requires two distinct signals (Bretscher and Cohn, 1970). Anergy is induced if B cells receive an activating signal by self-antigen but fail to receive another second signal within a given time frame. The second signal could be for example a TLR signal or T cell help mediated by CD40 ligand (CD40L) - CD40 interactions (Yarkoni et al., 2010). The functional consequences of anergy are reduced life-span possibly mediated by competition for B cell-activating factor (BAFF) (Lesley et al., 2004) and higher levels or the pro-apoptotic protein B cell leukaemia/lymphoma 2-like 11 (BCL2L11), also known as BCL2-interacting mediator of cell death (BIM) (Oliver et al., 2006) or induction of apoptosis by CD95-CD95L interaction with $CD4^+$ T cells (Rathmell et al., 1995).

If immature B cells have a weakly self-reactive BCR, the mechanism of clonal ignorance can maintain tolerance. Clonal ignorance of weakly autoreactive B cell clones is mediated by inhibitory BCR signalling molecules such as CD22, which increase the activation threshold of B cells (O'Keefe et al., 1999). If a self-antigen that is physically linked to a foreign antigen is recognised, endocytosed and displayed by a self-reactive B cell to a T cell cognate for the foreign antigen, an autoimmune reaction could occur as the T cell would give help to the B cell. This can be stopped by a higher BCR signalling threshold preventing B cells from

becoming activated and consequently preventing B cell migration to the T/B border, thus precluding T cell help (Pillai et al., 2011).

Avoidance of self-reactivity is important but a functional BCR must not only be non-reactive to self-antigen but also be able to produce a positive selection signal. It is thought that for positive selection at the immature B cell stage the BCR is involved in delivering a potentially ligand-independent, tonic survival signal to immature B cells. Indeed, loss of a functional BCR leads to rapid cell death of immature and mature B cells (Kraus et al., 2004; Lam et al., 1997) and presence of a functional BCR, composed of IgH, IgL, Igα and Igβ chains, already leads to some phosphorylation of downstream signalling molecules (Wienands et al., 1996). Functional immunoreceptor tyrosine-based activation motifs (ITAMs) of both essential signalling components Igβ and Igα are required for normal development of B cells (Kraus et al., 2004; Kraus et al., 1999; Reichlin et al., 2001). Remarkably, a fusion protein of Igα and Igβ delivered to the cell surface could also sustain B cell development in the absence of a complete BCR (Bannish et al., 2001). Interestingly, it was recently suggested that the surface BCR, in particular Igα and Igβ, might only serve as a signalling scaffold for delivery of the tonic survival signal. It was shown that BAFF binding to the BAFF receptor (BAFFR) leads to Igα and Igβ phosphorylation and recruitment of SYK, which then transduces the survival signal. In this model the tonic signal would therefore originate from BAFFR engagement and would be independent of a ligand for the BCR. However, there could also be a requirement for two distinct signals,

both of which are required for B cell survival: A (tonic) BCR signal and a BAFF-induced signal (Schweighoffer et al., 2013). In support of the former hypothesis, BAFFR expression directly correlates with tonic BCR signalling as assessed by RAS-driven ERK activation (Rowland et al., 2010a; Rowland et al., 2010b).

In the next step of B lymphopoiesis, selected, immature B cells migrate to peripheral lymphoid organs such as the spleen and lymph nodes to complete their development.

1.2.3 B cell development in peripheral compartments

Further maturation of immature B cells is necessary for functional competence since immature B cells respond with cell death to antigen encounter instead of activation and proliferation (Norvell et al., 1995). In the spleen, these immature B cells are called transitional B cells and divided into transitional stage 1, 2 and 3 (T1, T2, T3). As opposed to mature B cell subsets in the spleen, transitional B cells all express CD93 and are split up by the expression of IgM and CD23 into IgM^{high} $CD23^{-}$ T1, IgM^{high} $CD23^{+}$ T2 and IgM^{low} $CD23^{-}$ T3. The T2 stage can also be further subdivided into CD21 high marginal zone precursors (Allman et al., 2001). At this stage cells start to depend on BAFF (Batten et al., 2000; Schiemann et al., 2001) and upregulate IgD. For transition to the T3 stage signalling by Bruton's tyrosine kinase (BTK), a kinase found in the BCR signalling pathway, seems to be partially required. Moreover, there seems

to be on-going selection as cells are lost at the T1/T2 and the T2/T3 transition (Allman et al., 2001; Allman and Pillai, 2008).

The T3 stage has been suggested to contain a high amount of anergic B cells and may not constitute a developmental subset although the evidence so far is not clear (Merrell et al., 2006).

Interestingly, it has also been proposed that B cells can also continue maturing in the bone marrow. This idea is supported by the fact that the mature recirculating B cell pool in the bone marrow is functionally heterogeneous and a T2-like stage has been described. As in the periphery these B cells start responding to BAFF, upregulate IgD and CD23 and it is thus likely that B cells leave the bone marrow as immature or semi-mature B cells (Lindsley et al., 2007).

1.2.4 Mature B cell populations

A major peripheral site of B cell maturation is the spleen. The spleen is an encapsulated organ, split by trabeculae and is functionally divided into the red pulp and the white pulp.

The red pulp filters blood and is also involved in the removal of old erythrocytes and iron recycling. Arterial blood arrives in the cords of the red pulp, which do not have an endothelial lining. They are specialised structures consisting of fibroblasts, reticular fibres and macrophages. The blood then passes through a mesh of stress fibres into venous sinuses with endothelial lining which filters out old erythrocytes that are then phagocytosed by macrophages from the cords (Mebius and Kraal, 2005).

In addition, the red pulp is a site where plasmablasts and plasma cells are retained (Sze et al., 2000).

The white pulp is the lymphoid part of the spleen and resembles lymph nodes. It forms a lymphoid sheath around the branching arterioles. Closest to and surrounding the vessels is the T cell zone or periarteriolar lymphoid sheath (PALS). Follicular B cells (FOB) are found in the adjacent follicles in which germinal centres (GCs) are formed (Mebius and Kraal, 2005). GCs are the sites of SHM and CSR and the selection of the highest-affinity B cell clones (Victora and Nussenzweig, 2012). Surrounding the follicles and PALS is the marginal zone, which separates the white pulp from the red pulp. In it, marginal zone B cells (MZB), marginal zone macrophages and marginal zone metallophilic macrophages can be found. The marginal zone contains marginal zone sinuses through which part of the arterial blood flows in the direction of the red pulp. Towards the red pulp reticular fibroblasts, which are continuous with fibroblast in the red pulp, form the basis of the marginal zone together with sinus lining cells. MZB, DCs and marginal zone macrophages are found interspersed in this region. Towards the white pulp a layer of sinus-lining cells separates the marginal zone from the white pulp. Underneath these sinus-lining cells a layer of marginal zone metallophilic macrophages is located which then borders the PALS or follicles of the white pulp. Lymphocytes enter the white pulp through the marginal zone. In contrast, lymph nodes which otherwise have a very similar structure to the white pulp of the spleen do not contain a marginal zone. Here, lymphocytes enter mostly via high endothelial

venules located in the T cell zone or through afferent lymphatic vessels. Whereas in lymph nodes cells leave through efferent lymphatic vessels this process is currently not clearly defined in the spleen but evidence suggests that B cells leave via the marginal zone into the red pulp and then into the circulation whereas T cells probably avoid the marginal zone and pass into the red pulp via marginal zone bridging channels that directly connect PALS with the red pulp (Cyster and Schwab, 2012; Mebius and Kraal, 2005; Mueller and Germain, 2009).

In the spleen transitional B cells either mature to FOBs or MZBs. FOBs constitute the majority of mature B cells and can be even further subdivided into surface IgM^{low} $CD21^{low}$ FOB I and surface IgM^{high} $CD21^{mid}$ FOB II which have been suggested to be functionally distinct. FOB I represent two thirds of FOBs whereas FOB II constitute the remaining one third. In contrast to FOB I, FOB II develop independently of antigen, BTK and NOTCH signals. FOB II are thought to potentially be a reservoir for repopulating the MZB compartment during infections (Cariappa et al., 2007).

MZBs are located in the marginal sinuses of the spleen at the border of the white pulp and are retained there by the action of sphingosine-1-phosphate (S1P) binding to S1P receptor 1 (S1PR1) (Cinamon et al., 2004; Pillai and Cariappa, 2009). They interact with immature dendritic cells (DCs), which provide them with antigens (Balázs et al., 2002; Ferguson et al., 2004). They are particularly responsive to T-independent antigens respond rapidly to blood-borne pathogens and are highly mobile

(Pillai and Cariappa, 2009). Additionally, since the marginal zone is a site of blood flow with easy access for sampling antigens in circulation, MZBs help shuttle antigen to the follicle. This process is dependent on CXCR5 for movement towards the follicle and S1PR1 and S1PR3 (Arnon et al., 2013; Cinamon et al., 2008). MZBs express high levels of CD1d, which is a molecule for presentation of lipid antigens by APCs. MZB have been shown to instruct invariant natural killer cells (iNKT) via CD1d which in turn provide help to the MZB in the form of CD80, CD86, IFNγ and CD40L but not IL-4 (Leadbetter et al., 2008).

It has been noted that development of transitional B cells into either FOBs or MZBs depends on different signalling molecules. Mutations that influence lineage choice of MZB versus FOB development are generally categorised into mutations affecting BCR signalling strength, BAFFR signalling, nuclear factor κ-light chain-enhancer of activated B cells (NFκB) signalling, NOTCH-pathway signalling and chemokine and integrin signalling (Pillai and Cariappa, 2009). For example, *Btk*-deficient mice have strongly reduced numbers of FOB I (Hardy et al., 1983) as do mice with mutations in phospholipase C γ 2 (PLCγ2) which is signalling downstream of BTK (Hikida et al., 2003; Pillai and Cariappa, 2009; Wen et al., 2003). Loss of the positive BCR signalling regulator CD45 also leads to loss of FOB I but was restored if the negative BCR signalling regulator SH2 domain-containing protein tyrosine phosphatase 1 (SHP1) was also deleted (Pani et al., 1997). In general, data indicates that lower BCR signalling favours development of MZB whereas stronger BCR signals

favour development into FOBs. In support of this, marginal zone precursor cells (MZP) were reduced in *Aiolos*-deficient mice, which have increased BCR signalling (Cariappa et al., 2001). Upon loss of the BCR signalling inhibitory molecule CD22 similar results were obtained (Samardzic et al., 2002). Several other B cell signalling component-deficient mice and transgenic BCR mouse models support this hypothesis (Pillai et al., 2005). BAFFR signalling and NFκB signalling are also critical for the MZB versus FOB B cell lineage decision. NFκB activation can be achieved by two different pathways using different NFκB subunits. These pathways are referred to as the canonical and the non-canonical NFκB pathway. Canonical NFκB signalling is required for development of MZBs but not FOBs (Cariappa et al., 2000). It was noted that although BAFFR signalling can elicit both canonical and non-canonical NFκB signalling it mainly activates non-canonical signalling and this was thought to be important for B cell survival (Claudio et al., 2002). Interestingly, BAFFR-deficiency, and thus development of both MZBs and FOBs, can be rescued by a constitutively active form of inhibitor of κ B kinase β (IKKβ), which elicits canonical NFκB signalling (Sasaki et al., 2006). These results suggest that BAFF-induced canonical NFκB signalling can substitute normal BAFF-induced non-canonical signalling and that non-canonical signalling is dispensable for MZB development. Besides, it was thought that tonic BCR signalling provides the non-canonical NFκB component p100 as a substrate for the processing by BAFF-induced signalling (Stadanlick et al., 2008). However, these results have to be evaluated in the light of BAFFR-

signalling through SYK (Schweighoffer et al., 2013) as in the previous study conclusions were based on the use of SYK inhibitors (Stadanlick et al., 2008). This suggests that BAFFR signals could suffice to provide p100 for processing via the non-canonical pathway.

Signalling by NOTCH2 has also been shown to be required for development of MZBs although the exact signalling mechanism is currently not clear. Mice lacking NOTCH signalling components like NOTCH2 (Saito et al., 2003), recombination signal binding protein for immunoglobulin kappa J region (RBP-Jκ) (Tanigaki et al., 2002), Mastermind-like 1 (MAML1) (Oyama et al., 2007; Wu et al., 2007) and the notch ligand Delta-like 1 (DL1) (Hozumi et al., 2004) all show reduced MZB compartments. Conversely, if NOTCH signalling is increased, mice have more MZBs such as in mice lacking the NOTCH signalling suppressor SPEN (also known as MINT) (Kuroda et al., 2003).

Several chemotactic, cell adhesion and motility genes also contribute to the formation of MZB in the marginal zone. As mentioned above S1P is the chemotactic ligand for S1PR1 that retains MZB in the marginal zone thus resisting the CXCL13 gradient for recruitment into the follicles mediated by CXCR5 (Cinamon et al., 2004; Cinamon et al., 2008). Furthermore, MZBs are held in the marginal zone by the integrin lymphocyte function-associated antigen 1 (LFA1) and $\alpha 4\beta 1$ integrins on MZBs binding to intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) expressed in the marginal zone. Integrins are downregulated by MZBs upon activation with LPS and

contribute to the relocalisation of the cells (Lu and Cyster, 2002). Wiscott-Aldrich syndrome protein (WASP), critical for regulation of cellular movement, is also important for MZB development. Cells deficient for WASP do not respond to S1P (Westerberg et al., 2008). RAC1 and RAC2, which promote actin polymerisation, have also been reported to be critical for normal numbers of MZB (Croker et al., 2002; Walmsley et al., 2003). Additionally, mutations of proteins related to integrin and BCR function or signalling are also linked to changes in the MZB compartment such as CD19, its downstream phosphoinositol signalling cascade including the PI3K isoform p110δ or the phosphatase and tensin homologue (PTEN) (Pillai and Cariappa, 2009).

In summary, the current model for the development of murine MZBs probably starts with weak BCR signalling in immature B cells, followed by DL1 and BAFF signals upon arrival in the spleen through the red pulp. Assuming that marginal zone precursors reside in the follicle, chemotactic signals then lead the transitional B cell to migrate towards the marginal zone, where they are retained by integrin signals. If the MZB compartment is full but cells do not have strong enough BTK signalling to commit to FOB I lineage they may develop into FOB II to serve as a reservoir in case MZB are rapidly depleted such as in malaria infection (Achtman et al., 2003; Pillai and Cariappa, 2009).

1.2.5 B1 B cells

In addition to the cells discussed above, which are generally referred to as B2 B cells, another B cell lineage exists in rodents termed B1 B cells with different developmental requirements and functions. In mice, the B1 B cells are firmly established and are characterised by lacking expression of CD23 and are separated into $CD5^+$ B1a B cells which constitute the majority of B1 B cells and $CD5^-$ B1b B cells (Baumgarth, 2010). In contrast, the existence of B1 B cells in human is currently under debate (Tangye, 2013). A human $CD5^+$ B cell subset does not seem to be the equivalent of mouse B1a B cells (Pillai and Cariappa, 2009). Human B1 B cells were characterised as $CD20^+$ $CD27^+$ $CD45^+$ (Griffin et al., 2011), however, studies showed that this population constitutes pre-plasmablasts leaving the possibility that it is either a mixed population with B1 B cells or just cells starting to commit to plasma cell differentiation (Covens et al., 2013; Tangye, 2013). Functionally, B1 B cells are more considered mediators of innate immunity as their antibodies often recognise repetitive epitopes such as carbohydrates and they are generally more self-reactive. In contrast, B2 B cells are generally thought to mediate adaptive immunity and form germinal centres and humoral immunity (Montecino-Rodriguez and Dorshkind, 2012).

With regards to the development of B1 B cells, two opposing theories exist. The “selection model” states that B1 B cells are selected by reacting with specific antigens (Haughton et al., 1993) whereas the “layered immune system” model proposed distinct progenitor cells for B1 B cells as

opposed to B2 B cells (Herzenberg and Herzenberg, 1989) which is currently supported by more evidence (Montecino-Rodriguez and Dorshkind, 2012).

B1 B cells were first identified in 1983 (Hayakawa et al., 1983) after a search for $CD5^+$ B cells was prompted by $CD5^+$ B cell lymphomas (Lanier et al., 1981). In support of the "layered immune system" theory a distinct $lineage^-$ $CD19^+$ $B220^{low\text{-}negative}$ B1 B cell progenitor cell has been identified (Montecino-Rodriguez et al., 2006).

B1 B cells create the first wave of B lymphocytes. They are thought to even precede HSCs as B1 B cells can be derived from unidentified progenitors in the yolk sac and para-aortic splanchnopleura at embryonic day 8.5 - 9.0 before HSCs appear on day 10.5 in the aorta-gonad mesonephros region. These unidentified progenitor cells give rise to the $lineage^-$ $CD19^+$ $B220^{low\text{-}negative}$ B1 progenitor cells that differentiate exclusively to B1 B cells and MZP (Yoshimoto et al., 2011).

B1 B cells can be produced by the adult bone marrow as well (Duber et al., 2009; Kikuchi and Kondo, 2006) however neonatal tissues are more effective at reconstituting B1 B cells. It was shown that there are distinct CLPs for B1 and B2 B cells and that the number of B1-producing CLPs decreases towards adulthood explaining why neonatal tissues perform better at the reconstitution (Barber et al., 2011).

It has been proposed that B1 B cell development also progresses though a transitional B cell stage (Casola, 2007), which is supported by the first wave of transitional B cells giving rise to mostly B1 B cells (Montecino-

Rodriguez and Dorshkind, 2011). However, there are differences to transitional B2 cells. B1 cells are BAFF-independent (Montecino-Rodriguez and Dorshkind, 2011; Sasaki et al., 2004; Schiemann et al., 2001) and their development occurs as long as either the canonical or the non-canonical NFκB pathway are functional whereas B2 B cells depend critically on the non-canonical pathway. B1 B cells can develop thought the transitional stage without canonical NFκB signalling but later seem to critically depend on canonical NFκB signalling for their maintenance (Montecino-Rodriguez and Dorshkind, 2011; Montecino-Rodriguez and Dorshkind, 2012). This dependence on canonical NFκB signalling is further supported by findings in other canonical NFκB signalling molecule-deficient mice (Thome, 2004). Other signalling molecules that have been reported to be critical for B2 B cell development have also been shown to be dispensable for B1 B cells including IL-7 (Carvalho et al., 2001) and PU.1 (Rosenbauer et al., 2006; Ye et al., 2005).

The current model suggests that B1 B cells are generated in several waves (Montecino-Rodriguez and Dorshkind, 2012). However, there might still be remaining plasticity between B1 and B2 B cells as B2 cells have been reported to acquire B1 cell phenotypes under certain conditions (Hastings et al., 2006). Moreover, B1 B cells turn over much more slowly than B2 B cells and are maintained mostly by self-renewal instead of constant influx from the bone marrow (Hao and Rajewsky, 2001). The self-renewal seems to be in part regulated by IgM levels as lower IgM levels induce more B1 B cells (Lalor et al., 1989).

Mature B1 B cells are predominantly located in the peritoneal and pleural cavities (Hayakawa et al., 1985). They have a different VDJ_H gene usage compared to B2 cells and fewer N nucleotides (Kantor et al., 1997; Tornberg and Holmberg, 1995). Their BCRs show increased specificities for self-antigens and pathogen-expressed molecules (Baumgarth, 2010). This repertoire is created in the absence of exogenous antigens (Haury et al., 1997). It is thought that the mild self-reactivity of the BCRs actually helps the cells in positive selection (Hayakawa et al., 1999) and it has been proposed that potentially all autoreactive BCRs cross-react with a foreign antigen and that thus only “useful” clones are selected (Baumgarth, 2010; Baumgarth et al., 2005). Interestingly, splenic and bone marrow B1 B cells are the major producers of the natural IgM antibodies whereas B1 B cells from coelomic cavities produce little (McIntyre et al., 1991). However, about 80 % of all serum IgM is estimated to come from B1 B cells (Baumgarth et al., 1999). There is ample evidence that natural antibodies contribute to defence against infection, acting directly at the barrier even before innate immunity. Protection mechanisms by natural antibodies include neutralisation, complement activation, inhibition of pathogen activation and potentially increasing adaptive responses by helping deposit antigen on follicular dendritic cells (FDCs) for antigen-presentation to B2 cells in a germinal centre reaction (Baumgarth, 2010). In addition, B1 B cells can class switch, particularly to IgA and thus participate in responses at mucosal surfaces as well (Kaminski and Stavnezer, 2006). Secretion of natural IgA by B1 B cells seems to be

dependent on early exposure during ontogeny to the commensal bacteria as challenge with individual commensal microbes in adult gnotobiotic mice resulted in B2 B cell-derived IgA responses (Thurnheer et al., 2003). Moreover B1 B cells can contribute to immune homeostasis by phagocytosis and promotion of phagocytosis of apoptotic debris via autoreactive IgM (Chen et al., 2009; Rodriguez-Manzanet et al., 2010). Overall, B1 B cells seem to be more responsive to secondary signals such as PRR engagement and less responsive to BCR activation compared to B2 B cells (Baumgarth, 2010; Martin et al., 2001).

In general B1a B cells are thought to mediate more innate immune responses by secretion of natural antibodies whereas B1b B cells mediate adaptive responses to particular antigens. For example CD19-deficient mice lack B1a B cells and are susceptible to infection with *Streptococcus pneumonia* because they lack natural antibodies functioning as first barrier. CD19-transgenic mice in contrast produce ample amounts of natural antibody and are protected. However, if mice were first immunised with pneumococcal polysaccharide, CD19-transgenic mice were not protected against lethal *Streptococcus pneumonia* challenge whereas CD19-deficient mice were protected due to higher numbers of B1b B cells (Haas et al., 2005). Other functions of B1b cells include protection against *Salmonella typhimurium* though antibodies against outer membrane proteins (Omps), in particular OmpF (Gil-Cruz et al., 2009) and against *Borrelia hermsii* by generating a T-independent IgM response that also

generated T-independent memory again emphasising the adaptive nature of B1b B cells (Alugupalli et al., 2004).

1.3 B Cell activation

B cells become activated following encounter of cognate antigen. Cross-linking of the BCR by the antigen induces downstream BCR signalling (Tolar and Spillane, 2014). B cells can be activated by soluble antigen; however, *in vivo* the size of the antigen is limited to about 70 kDa because bigger antigens are filtered out before reaching the follicle due to anatomic constraints (Gretz et al., 2000; Pape et al., 2007; Sixt et al., 2005). Bigger antigens are readily presented by APCs such as DCs (Bergtold et al., 2005; Heesters et al., 2013).

1.3.1 Early events in B cell activation

Upon B cell activation the BCR must change from a resting state into an activated state that induces downstream signalling. The natural conformation of the BCR on the surface of a resting B cell is currently controversial. Conflicting data point either to single molecules being present on the surface (Tolar et al., 2005) or the aggregation of BCRs into oligomers (Schamel and Reth, 2000; Yang and Reth, 2010). The overall organisation of BCRs on the cell surface seems to be of importance as BCRs on resting B cells are restricted in their diffusion. Disruption of this segregating network by interfering with the actin cytoskeleton leads to

BCR signalling and increased BCR mobility in the plasma membrane (Treanor et al., 2010).

If antigen is bound, BCRs form microclusters, which then initiate signalling by downstream kinases (Depoil et al., 2008). Two competing but not necessarily mutually exclusive theories explain how initial BCR signalling might be regulated (Harwood and Batista, 2010). The “conformational change model” predicts a change in the structure of a single BCR molecule upon antigen binding. The structural change is the signal transferred into the cell to activate the downstream signalling cascade. However, direct evidence for this model is still missing although it has been shown that BCRs have mechanosensory properties since they respond to the stiffness of the presented antigen and initial oligomerisation depends on the Cμ4 domain of the BCR (Tolar et al., 2009; Wan et al., 2013). The second model is called the “kinetic segregation model” in which bulky BCR signalling-inhibiting phosphatases such as CD45 and CD148 are excluded from the immunological synapse and thus allow signalling upon clustering of the BCR and formation of the central supramolecular activation cluster (cSMAC) (Harwood and Batista, 2010). Evidence for this model comes from the involvement of the cytoskeleton in the movement and segregation of BCR signalling molecules including IgD, IgM, CD19 and CD81 (Depoil et al., 2008; Mattila et al., 2013; Treanor et al., 2011).

After initial signalling, the BCR is internalised together with the antigen, which is then processed for presentation to T cells via MHC molecules (Lanzavecchia, 1985). The internalisation process again involves the

cytoskeleton and mechanically tests the affinity of the BCR for the antigen if the antigen is presented on the surface of an APC (Natkanski et al., 2013).

Apart from activation through the BCR, B cells can be activated by other signals such as CD40L, IL-4, TLR ligands or BAFF (Hua and Hou, 2013; Rush and Hodgkin, 2001; Schneider et al., 1999; Wykes, 2003). In response to activation by different stimuli, activation markers such as CD23, CD69, CD86 or MHC class II are differentially regulated often serving as a read-out to measure activation (Barr et al., 2007; Erlanson et al., 1998).

B cell activation results in differentiation of naïve B cells into antigen-experienced B cell subsets with effector functions such as plasma cells or memory B cells (Harwood and Batista, 2010). The main effector function of B cells is considered to be antibody production as fully differentiated plasma cells. In addition to antibody production, B cells have other effector functions often mediated though production of cytokines. These include enlargement of lymph nodes, hence facilitating other cell-cell interactions, support of Tfh cells in the germinal centre and regulation of immune homeostasis particularly in several autoimmune disorders (Angeli et al., 2006; Leon et al., 2012; Mauri and Bosma, 2012).

Cytokines produced by B cells include IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-16, IFNγ, Lymphotoxin α and β (LTα and LTβ), TGFβ and TNFα and vascular endothelial growth factor A (VEGF-A) which mediate several of

these effector functions (Angeli et al., 2006; Lund, 2008; Lund et al., 2005).

BCR ligands that lead to B cell activation can be classified into a number of different categories. Classically, BCR ligands have been divided into thymus (or T)-independent or T-dependent antigens depending on whether athymic mice can generate antibodies in response to the antigen (Vinuesa and Chang, 2013). T-independent antigens have been further subdivided into T-independent type 1 and T-independent type 2 antigens depending on whether CBA/N mice, which have an X-linked deficiency in the kinase BTK, which is important for BCR signal transduction, can respond to the antigen (Mond et al., 1995; Mosier et al., 1977). T-independent type 1 antigens are microbial ligands that activate PRRs such as TLRs and thus, in addition to the BCR signal, deliver a second activation signal via these receptors (Bekeredjian-Ding and Jego, 2009). T-independent type 2 antigens are typically multivalent antigens that extensively crosslink the BCR and provide a long, sustained signal transmitted via BTK leading to B cell activation (Mond et al., 1995).

More recently, antibody responses have been further divided, adding T-independent type 3 responses and splitting T-dependent responses into type 1 and type 2. T-independent type 3 responses are characterised by help to B cells by bone marrow-derived innate immune cells in response to mucosal or blood born bacteria instead of thymus-derived cells (Vinuesa and Chang, 2013). In this regard, it has been shown that a subset of neutrophils termed B cell helper neutrophils (N_{BH}) locate at the periphery

of the marginal zone and help MZB to perform SHM, CSR to IgG and IgA and secrete Ig dependent on N_{BH} secretion of BAFF, a proliferation inducing ligand (APRIL) and IL-21 (Puga et al., 2012). Other data indicate a similar mechanism for DCs and macrophages in providing help to B cells via BAFF or APRIL (Balázs et al., 2002; García de Vinuesa et al., 1999; Tezuka et al., 2007). Some data also suggests that mast cells, basophils and eosinophils can also provide help to B cells to elicit antibody responses (Gauchat et al., 1993; Merluzzi et al., 2010; Wang and Weller, 2008).

T-dependent antibody responses are subdivided by the type of antigen and the type of cell providing the help. T-dependent type 1 responses are the classical responses to protein antigen in which B cells interact with Tfh cells generating germinal centres and giving rise to long-lived plasma cells and memory B cells as well as short-lived extrafollicular plasma cells (Vinuesa and Chang, 2013). T-dependent type 2 responses are against microbial, synthetic or self-non-protein glycolipid antigens that can be presented by CD1d on B cells such as α-galactosylceramide (Venkataswamy and Porcelli, 2010; Vinuesa and Chang, 2013). Instead of interaction with Tfh cells, B cells are helped by natural killer T (NKT) cells to induce antibody responses (Barral et al., 2008; Chang et al., 2012; Detre et al., 2012; Leadbetter et al., 2008). NKT cells develop in the thymus and the response is consequently T-dependent (Godfrey and Berzins, 2007; Vinuesa and Chang, 2013). Antigen is taken up by DCs and presumably presented to NKT cells, which activates NKT cells in an

IL-12 and CD40 dependent manner (Kitamura et al., 1999). Similarly to Tfh, activated NKT T cells develop into follicular helper NKT (NKT_{FH}) cells together with B cells presenting the glycolipid antigen via CD1d. This process is dependent on BCL6 (Chang et al., 2012) and signalling lymphocyte activation molecule (SLAM)–associated protein (SAP) (Detre et al., 2012). NKT cell help to B cells is provided in the form of CD40-CD40L and CD28-CD80/CD86 interactions and leads to a rapid extrafollicular plasma cell response (Chang et al., 2012; Leadbetter et al., 2008). Although T-dependent type II responses generate germinal centres in which affinity maturation and CSR occur, they do not result in formation of long-lived plasma cells or humoral memory. Interestingly, both the extrafollicular plasma cell response and the germinal centre formation depend on NKT-derived IL-21 (Chang et al., 2012; King et al., 2012).

1.3.2 The germinal centre reaction

Germinal centres are the sites of SHM and affinity-based selection of B cells (Berek et al., 1991; Jacob et al., 1991). They are separated into a light zone and a dark zone (Nieuwenhuis and Opstelten, 1984). The light zone was originally shown to be the site of antigen retention on follicular dendritic cells located there and the dark zone was shown to contain proliferating cells (Mandel et al., 1980; Mandel et al., 1981). It is now clear that cells shuttle between these two zones, proliferate in the dark zone and then migrate to the light zone and back (MacLennan, 1994; Victora et al., 2010). Germinal centres are open structures where B cells can enter and

leave and interact with FDCs and T cells. Germinal centre B cells in the dark zone are called centroblasts and are bigger and proliferating while germinal centre B cells in the light zone are called centrocytes, which are smaller and interact with T cells to compete for selection (Allen et al., 2007a; Allen et al., 2007b; Hauser et al., 2007; Schwickert et al., 2007). However, cells also proliferate in the light zone to a certain degree and the cell proliferation programme is already initiated in the light zone (Allen et al., 2007a; Bannard et al., 2013; Victora et al., 2010). As dark zone cells are proliferating they display a higher nucleus to cytoplasm ration and thus appear darker in light microscopy (Nieuwenhuis and Opstelten, 1984). Phenotypically GCB are IgD^{-}, high in surface n-glycolylneuraminic acid which is bound by the antibody clone GL7, high for CD95 expression, high in binding peanut agglutinin (PNA) and CD38 expression is reduced on GCB in mice but elevated in humans (Victora and Nussenzweig, 2012). GCB can further be subdivided into $CXCR4^{high}$ $CD83^{low}$ $CD86^{low}$ centroblasts and $CXCR4^{low}$ $CD83^{high}$ $CD86^{high}$ centrocytes (Victora et al., 2010).

Apart from germinal centre B cells, these structures contain several other cell types. A small number of IgD^{+} naïve B cells transiting through the GCs have been reported. Newly-activated B cells either seed germinal centres or reuse old ones potentially from an unrelated immune response. Rarely, they can also be entered by exceptional, naïve high-affinity clones which directly enter affinity maturation (Schwickert et al., 2009; Schwickert et al., 2007).

The light zone contains a network of FDCs and T cells (Lindquist et al., 2004). Conventional DCs are found as well which might serve to stimulate T cells (Grouard et al., 1996), however, their function is currently elusive (Lindquist et al., 2004). FDCs serve to display antigen to GCB undergoing affinity maturation and display antigen as iccosomes or immune complexes on their surface (Szakal et al., 1985; Szakal et al., 1988).

Furthermore, tingible body macrophages are found in the light zone. These cells are thought to take up apoptotic bodies of B cells that failed selection. Tingible body macrophages mainly serve in the downregulation of the germinal centre reaction as defective signalling in this population leads to enhanced GC responses (Rahman et al., 2010; Victora and Nussenzweig, 2012).

Another population of major importance in the GC are T cells, in particular Tfh. Tfh are the major cell type providing help to GCB (Ramiscal and Vinuesa, 2013). Their development begins at the T:B border after encounter of B cells displaying cognate antigen. After a prolonged encounter with the B cell, antigen-specific T cells start to upregulate BCL6, CXCR5 and PD-1. They precede B cells in the migration to the follicle to establish the GC. (Kerfoot et al., 2011). Generation of Tfh also depends on BCL6, IL-21, and inducible co-stimulator (ICOS) and SAP (Linterman et al., 2010; Nurieva et al., 2008; Nurieva et al., 2009; Qi et al., 2008; Vogelzang et al., 2008). Interestingly, GCB and Tfh mutually depend on each other for survival and keep the GC reaction going by expression of IL-21 by T cells and ICOS ligand (ICOSL) by B cells (Nurieva et al., 2008;

Vogelzang et al., 2008). The main stimuli used by Tfh to regulate GCB affinity maturation and survival are CD40L and IL-21 (Han et al., 1995; Linterman et al., 2010). As mentioned above, in addition to Tfh, also NKT cells can serve as B cell helpers when differentiating into NKT_{FH} cells in the case T-dependent type 2 antigens (Ramiscal and Vinuesa, 2013; Vinuesa and Chang, 2013).

Moreover, follicular regulatory T cells (Tfr) have been identified in the germinal centre. These cells are $CD4^+$ $CD25^{+/-}$ $CXCR5^{high}$ $PD\text{-}1^{high}$ $Foxp3^+$ $BCL6^+$ $BLIMP1^+$ and depend on SAP and BCL6 much like Tfh and seem to arise from nTregs although generation from pTregs has not been formally excluded. They regulate Tfh and GCB numbers and lack of Tfr leads to increased non-antigen-specific GCB and bigger GCs (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Furthermore, a distinct $CD8^+$ Treg lineage has been described that is critical for maintenance of self tolerance via targeting Tfh expressing the MHC class I molecule Qa-1. Disruption of the interaction of these $CD8^+$ Tregs with $Qa\text{-}1^+$ Tfh leads to a systemic lupus erythematosus-like autoimmune disorder in mice (Kim et al., 2010).

The current model of selection in the germinal centre is that GCB cycle between light zone and dark zone (Victora and Mesin, 2014). In the dark zone cells proliferate and upregulate AID to perform SHM and CSR (Victora and Mesin, 2014; Victora and Nussenzweig, 2012). After around an average of two divisions cells return to the light zone to test their mutated BCRs for affinity (Gitlin et al., 2014). Mutated GCB clones then

collect antigen from FDCs; the higher the affinity the more antigen can be endocytosed and subsequently displayed as pMHC by a given clone. GCB then compete for help by Tfh (Gitlin et al., 2014; Schwickert et al., 2011; Victora et al., 2010). Tfh interact with GCB in transient large contacts and interact with both selected and non-selected cells (Shulman et al., 2014). The more antigen a B cell presents via pMHC to a cognate Tfh, the more likely it is that the B cell will receive T cell help for survival. Successful clones initiate S phase in the light zone and return to the dark zone to proliferate and start another round of selection. The variable number of GCB cell divisions initiated in a round of selection seems to be controlled by Tfh (Gitlin et al., 2014; Schwickert et al., 2011; Victora et al., 2010).

As the process of selection in the germinal centre is becoming more and more well-defined, several other questions about germinal centres remain unanswered. The regulation of how an activated B cell becomes either an extrafollicular plasma cell or a GCB is currently obscure. Reports on the involvement of BCR signalling strength through antigen affinity are not clear cut but some data suggests that low affinity clones preferentially become GCB whereas high-affinity ones differentiate into extrafollicular plasma cells (Paus et al., 2006; Shih et al., 2002; Zotos and Tarlinton, 2012). Signals that lead to exit from the germinal centre and differentiation into memory B cells or plasma cells are currently elusive too. However, there is evidence for a temporal divide between those to cell fates with memory B cells generally arising earlier and having fewer mutations whereas long-lived plasma cells are produced later in the response,

showing more mutations and higher affinity (Shlomchik and Weisel, 2012; Smith et al., 1997; Takemori et al., 2014). Furthermore, it is a conundrum how negative selection of autoreactive GCB clones that also cross-react with foreign antigen is achieved. These cells could potentially be positively selected by Tfh cells and thus lead to autoimmunity. Tfr have arisen as a possible candidate population to be responsible for this phenomenon (Ramiscal and Vinuesa, 2013).

1.3.2.1 Somatic hypermutation

Germinal centres are the sites of SHM. SHM is the second mechanism after VDJ recombination by which antibody variable genes are diversified. SHM is the mechanism that introduces mutations into the V(D)J regions of the IgH and IgL loci (Matthews et al., 2014). Mutations occur at a rate of 1 in 100 – 1000 base pairs per generation (McKean et al., 1984) and occur along the entire variable region but preferentially at RGYW / WRCY (R = A / G, Y = C / T, W = A / T) motifs which is due to the enzyme AID (Rogozin and Diaz, 2004). AID is specifically expressed in germinal centre B cells (Muramatsu et al., 1999) and is required for SHM as well as for CSR and gene conversion (Harris et al., 2002; Muramatsu et al., 2000). The region that is subject to mutation stretches from 100 – 200 bp downstream of the promoter region of the V genes to 1.5 – 2 kB further downstream (Lebecque and Gearhart, 1990; Rada et al., 1994) and requires transcription of these regions (Peters and Storb, 1996). SHM is generally divided into two phases. The first phase starts with AID

deaminating deoxycytidine (dC) to deoxyuridine (dU) at dC:deoxyguanosine (dG) base pairs (Petersen-Mahrt et al., 2002; Rada et al., 1998). This can lead to transition mutations via replication over the dU:dG pair, termed phase 1A. Alternatively, dU can be removed by base excision repair enzymes, mainly uracil DNA glycosylase (UNG) but also by single-strand selective monofunctional uracil DNA glycosylase (SMUG1), thus creating an abasic site (Di Noia and Neuberger, 2007; Dingler et al., 2014; Rada et al., 2002). This site can give rise to all four bases if replicated and preference of the inserted base would depend on the polymerase. This process is called phase 1B. Otherwise, normal repair would involve removal of the whole nucleotide by apyrimidic endonuclease and normal template-based correction back to dC by a DNA polymerase. Phase 2 is the main pathway for creation of mutations at adenosine (A) : thymidine (T) pairs. Here a dU:dG mismatch can also be recognised by the mismatch repair machinery which nicks the DNA around the mismatch site and excises it, usually a stretch of around 30 – 100 bp in length. The error prone DNA polymerase η then could create more mutations at this site at A:T pairs (Di Noia and Neuberger, 2007; Petersen-Mahrt et al., 2002).

1.3.2.2 Class switch recombination

CSR is the mechanism for exchanging the constant region of the Ig-heavy chain i.e. Cμ for a Cγ, Cα or Cε region. Thereby expression is switched from IgM to IgG, IgA or IgE respectively. Eight C_H genes comprise the

C57BL/6 mouse IgH locus: Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2c, Cε and Cα (Matthews et al., 2014; Zhang et al., 2012). In the recombination process, upstream C_H genes of the constant region to be exchanged are deleted. All mouse C_H genes are preceded by a switch (S) region with the exception of Cδ, which is usually simultaneously expressed through differential splicing with Cμ and creates double-positive IgM^+ IgD^+ B cells (Matthews et al., 2014; Moore et al., 1981). Switch regions are required for CSR (Shinkura et al., 2003) and need to be germline transcribed. Each constant region with the exception of Cδ is comprised of a cytokine inducible promoter, an intervening (I)-exon, the S region and the respective coding exons for the C_H region. I, S and C_H exons are transcribed, and the S region is spliced out creating a non-translated, sterile, germline transcript (Lennon and Perry, 1985; Matthews et al., 2014). Cμ germline transcription is constitutive and transcription of a particular downstream C_H gene leads to switching to this isotype. Which isotype is switched to depends on the stimulus. In mice, LPS leads to IgG2b and IgG3 switching, IL-4 to IgG1 and IgE switching, BAFF signalling to IgG and IgA switching, and TFGβ to IgA switching (Litinskiy et al., 2002; Lutzker et al., 1988; Rothman et al., 1988; Stavnezer et al., 1988). Transcription of the germline transcripts leads to formation of RNA that stays bound to the template strand due to high GC content of the region. The single-stranded non-template strand forms an R-loop, which serves as a template for AID (Chaudhuri et al., 2003; Matthews et al., 2014; Shinkura et al., 2003). Similar to SHM, AID deaminates dCs on single

stranded DNA (Chaudhuri et al., 2003; Pham et al., 2003) and two pathways can lead to the required double strand break (DSB) for the recombination to occur. The main pathway seems to be via UNG, which generates an abasic site followed by generation of a nick by apurinic/apyrimidinic endonuclease 1 (APE1). Two similar nicks, close but on opposite strands, generate a staggered DSB. Alternatively, mismatch repair can recognise the dU:dG mismatch, generate a nick and digest a stretch to create single-stranded DNA. Again two such events on opposite strands can create a DSB (Matthews et al., 2014; Rada et al., 2004). DSBs are then repaired by the classical non-homologous end joining pathway or the alternative end joining pathway, bringing together the variable region with a new C_H region and excising the intervening DNA between participating S regions into a circle called the switch circle (Iwasato et al., 1990; Matsuoka et al., 1990; Matthews et al., 2014).

1.3.3 Plasma cells

Plasma cells are the terminally differentiated B cell subset that produces antibodies. There are two ways in which a plasma cell can be generated. The first possibility is that plasma cells arise in an extrafollicular response, which is generally faster. The second way is that plasma cells arise in the follicle from germinal centres, which generally creates long-lived plasma cells (Oracki et al., 2010). Plasma cells can be phenotypically identified by high expression of CD138 (syndecan-1) (Sanderson et al., 1989). B lymphocyte-induced maturation protein 1 (BLIMP1), encoded by the

Prdm1 gene, is the signature transcription factor of plasma cells (Shaffer et al., 2002; Turner et al., 1994). Developing plasma cells are termed plasmablasts. Expression levels of BLIMP1 identify different stages of plasma cell development. Low BLIMP1 levels are found in plasmablasts, intermediate levels in short-lived plasma cells and high levels in plasma cells (Kallies et al., 2004). BLIMP1 regulates the plasma cell fate together with the transcription factors IRF4 and XBP1 counteracting the transcription factors for naïve or GCB fate decisions including PAX5, BCL6 and metastasis-associated 1 family, member 3 (MTA3) and BTB and CNC homology 1, basic leucine-zipper transcription factor 2 (BACH2) (Dent et al., 1997; Fujita et al., 2004; Hwangbo et al., 2004; Lin et al., 2004; Nutt et al., 2011; Shapiro-Shelef and Calame, 2005). Interestingly, similar to PAX5 for B lineage commitment, BLIMP1 is not the initiator of plasma cell development, as upregulation of several other plasma cell-related genes precede *Blimp1* transcription. Moreover, chimeras of *Rag1*-deficient mice reconstituted with foetal liver *Prdm1*-deficient bone marrow also have serum antibodies (Kallies et al., 2007).

Extrafollicular antibody responses are the main pathway in response to T-independent antigen and are often, but not exclusively, initiated by marginal zone or B1 B cells (Lane et al., 1986; MacLennan et al., 2003; Martin et al., 2001; Oliver et al., 1997; Vinuesa et al., 2003). Although T-independent antigens mostly lead to an extrafollicular antibody response, abortive germinal centres have been reported as well (de Vinuesa et al., 2000; Shih et al., 2002). In the case of T-dependent responses, which can

generate such extrafollicular foci as well, it has been shown that OX40-OX40L interactions and IL-21 by T cells are important for the development of extrafollicular foci (Odegard et al., 2008; Stüber and Strober, 1996). Extrafollicular responses only generate short-lived plasma cells as these cells rapidly disappear after the response (Smith et al., 1996).

T-dependent responses generally lead to the generation of germinal centres. Plasma cells arising from this pathway are generally high-affinity and long-lived and reside in the bone marrow (Oracki et al., 2010). As discussed earlier the signals leading to the differentiation of plasma cells from GCB are not clear but might involve an affinity threshold and instructions by T cells (Paus et al., 2006; Phan et al., 2006; Smith et al., 1997). These cells home from the follicle to either the splenic red pulp or the bone marrow, dependent on several chemokines. Lack of S1PR1 has been shown to reduce numbers of antibody secreting cells (AFCs) in the blood and bone marrow (Kabashima et al., 2006). Similar results were obtained upon deficiency of the S1PR1 regulator, Krüppel-like factor 2 (KLF2) (Winkelmann et al., 2011). Expression of CXCL12 is found in the bone marrow and splenic red pulp. Plasma cells upregulate CXCR4 to respond better to CXCL12 whereas they downregulate CXCR5 and CCR7 to stop responding to CXCL13 in the follicle and to CCL19 and CCL21 in the T cell zones leading to their movement into the bone marrow or red pulp (Hargreaves et al., 2001). A niche in the bone marrow seems to support the survival of a limited number of long-lived plasma cells helping to keep antibody serum levels constant (Radbruch et al., 2006). The

survival niche in the bone marrow is not completely defined yet but is comprised of VCAM1 and CXCL12-expressing stromal cells (Tokoyoda et al., 2004) and recently identified APRIL and IL-6-producing eosinophils (Chu and Berek, 2013; Chu et al., 2011). The maintenance of plasma cells is independent of cognate antigen (Manz et al., 1998).

1.3.4 Memory B cells

Together with long-lived plasma cells, which maintain serum levels of antibodies against previously encountered antigens, memory B cells form the basis of humoral, immunological memory (Takemori et al., 2014). Upon restimulation these cells can quickly differentiate into AFCs or generate new germinal centres to produce even higher affinity clones (Dogan et al., 2009). Several reasons have been put forward to explain the faster kinetics of secondary responses compared to a primary responses. Firstly, there is a higher precursor frequency of antigen-specific cells in a secondary response making early activation of antigen-specific cells more likely. Secondly, memory B cells are strategically located at sites of antigen drainage such as the marginal zone and memory B cells seem to be generally maintained in the spleen (Mamani-Matsuda et al., 2008; Tangye and Tarlinton, 2009). Thirdly, they, at least partially, have higher affinity BCRs due to SHM. Fourthly, again at least partially, memory B cells have switched BCR isotypes, which might confer better signalling properties compared to an IgM or IgD BCR. Fifthly, they constantly express higher levels of the co-stimulatory molecules CD80 and CD86

(Surova and Jumaa, 2014; Tangye and Tarlinton, 2009) and lastly, they have an altered transcriptional profile suggesting that memory B cells are poised for activation (Bhattacharya et al., 2007; Tomayko et al., 2008).

Studies on memory B cells have been hampered by a lack of consensus on the definition of B cell memory. Moreover, it has recently become clear that memory B cells are heterogeneous and they have been divided up by several systems including expression of antibody isotypes (Dogan et al., 2009; Pape et al., 2011; Taylor et al., 2012a; Taylor et al., 2012b; Weill et al., 2013), dependence or independence on the germinal centre reaction (Good-Jacobson and Tarlinton, 2012; Kaji et al., 2012; Shlomchik and Weisel, 2012; Takemori et al., 2014; Taylor et al., 2012b; Weill et al., 2013) or the expression of surface markers rather unspecific for murine memory B cells including CD80, programmed cell death ligand 2 (PD-L2), CD73 and CD35 (Anderson et al., 2007; Shlomchik and Weisel, 2012; Tomayko et al., 2010; Zuccarino-Catania et al., 2014).

It was shown that memory B cells can form in response to T-dependent immunisation without the need for germinal centres. As expected these germinal centre-independent memory B cells have fewer mutations than germinal centre derived ones (Kaji et al., 2012; Toyama et al., 2002). In addition, memory B cells have a unique transcriptional profile. As these cells seem to self-renew, they share transcriptional similarities with HSCs (Bhattacharya et al., 2007; Ehrhardt et al., 2008; Klein et al., 2003; Luckey et al., 2006; Tomayko et al., 2008).

Functional differences have been ascribed to the different subsets using cell surface markers and antibody isotypes. For example, it was initially reported that IgM^+ memory B cells differentiate into new germinal centres whereas IgG^+ memory B cells become AFCs (Dogan et al., 2009). Similarly, it was proposed that IgM^+ memory B cells constitute a long-lived reservoir that sustains the memory pool whereas switched Ig memory B cells respond rapidly and initially keep antibody serum levels high (Pape et al., 2011). More recently it was shown that $CD80^+$ $PD\text{-}L2^+$ memory B cells produce AFCs but not germinal centres and $CD80^-$ $PD\text{-}L2^-$ memory B cells produce germinal centres but not AFCs independent of the antibody isotype. In relation to the previous studies it should be noted though that switched memory B cells (defined as IgM^- in this study) were 73 % $CD80^+$ $PD\text{-}L2^+$ and unswitched (i.e. $IgG1^-$) memory B cells were 45 % $CD80^-$ $PD\text{-}L2^-$ but also contained 15 % $CD80^-$ $PD\text{-}L2^+$ and 27 % $CD80^+$ $PD\text{-}L2^+$ cells (Zuccarino-Catania et al., 2014).

In summary, several types of memory B cells exist and their different contributions to various aspects of humoral immunity still need to be evaluated in more detail. Reliable surface markers to define distinct memory B cell subsets would substantially facilitate research and make results easier to compare. Nevertheless, the contribution of B cell memory to a protective immune response against repeated pathogen challenge and in vaccination is indispensable (Good-Jacobson and Tarlinton, 2012; Takemori et al., 2014).

1.3.5 Regulatory B cells

B cells not only participate in proinflammatory reactions but also have roles in immune homeostasis and the downregulation of immune responses (Vaughan et al., 2011). It was initially suggested that there is a type of suppressive B cell derived from delayed-type hypersensitivity studies (Katz et al., 1974; Neta and Salvin, 1974). Later it was shown that B cell-deficient mice have reduced recovery from experimental autoimmune encephalomyelitis (EAE) (Wolf et al., 1996) and that B cells can prevent colitis in the context of TCRα-deficient mice (Mizoguchi et al., 1997). It was later shown that B cell-derived, immunosuppressive IL-10 was necessary for protection against EAE (Fillatreau et al., 2002; Mauri et al., 2003; Saraiva and O'Garra, 2010).

In addition to IL-10, several other immunosuppressive mechanisms of regulatory B cells (Bregs) have been identified. Thus, the gold standard for their identification is suppressive function upon transfer although production of IL-10 is very often used for identification as well (Mauri and Bosma, 2012; Rosser et al., 2014). Bregs can be subdivided into a number of subsets although their origins and relations to each other are not completely clear. In the mouse T2-MZP cells could be adoptively transferred and had immunosuppressive functions (Blair et al., 2009; Carter et al., 2012; Evans et al., 2007). Marginal zone B cells have also been reported to suppress immune responses to *Leishmania donovani* and have been implicated in tolerance to apoptotic cells (Bankoti et al., 2012; Gray et al., 2007; Miles et al., 2012). Moreover, IL-10-producing

$CD138^+$ plasmablasts have been identified in a *Salmonella typhimurium* infection model (Neves et al., 2010). Another subset of IL-10 producing, $CD1d^{hi}$ $CD5^+$ B cells, termed B10 cells, was found to ameliorate T-dependent inflammation (Yanaba et al., 2008). B10 cells have been proposed to have dedicated progenitors called B10pro, which depend on a particular BCR specificities as they are strongly reduced in BCR transgenic mouse models (Kalampokis et al., 2013; Yanaba et al., 2009). These cells have so far only been identified by the production of IL-10 upon appropriate stimulation as all other markers overlap with normal B cell populations (Kalampokis et al., 2013).

Several cellular targets by which Bregs mediate their homeostatic function have been identified. For example, Bregs can increase Treg numbers (Carter et al., 2012; Sun et al., 2008). Cognate interactions with $CD4^+$ T cells have also been shown to be essential for suppression and depend on CD40 - CD40L interactions and IL-21 (Yoshizaki et al., 2012). Breg-derived IL-10 can also regulate $CD8^+$ T cells (Bankoti et al., 2012; Schioppa et al., 2011). IL-10 secretion by Bregs can reduce IL-12 production by DCs and thus reduce Th1 development (Sun et al., 2005; Zhang et al., 2007). Enhanced potential to phagocytose was also shown for macrophages in the absence of Bregs (Horikawa et al., 2013). Neutrophils too can also be regulated by Bregs, as they are found in higher numbers in mice with reduced numbers of Bregs (Neves et al., 2010). Furthermore, Bregs can also regulate B cell responses themselves either in a direct or indirect fashion. Many models find reduced

autoantibody levels following Breg-mediated suppression (Evans et al., 2007; Gray et al., 2007; Mauri et al., 2003; Miles et al., 2012).

Due to the increasing amount of data on Breg-mediated suppression, their functional mechanisms have also been categorised. Bregs can either mediate suppression through direct cognate antigen-mediated interactions, direct cell-contact without cognate interactions, bystander suppression via cytokines, or indirect suppression via APCs or Tregs (Rosser et al., 2014).

1.4 Signalling pathways in B cells

Several B cell signalling pathways have been mentioned throughout the text. I will briefly discuss the most salient features of their downstream signalling cascades.

1.4.1 B cell receptor signalling

As discussed earlier, cross-linking of BCR leads to B cell activation via BCR signalling. The main signalling pathways subsequent to BCR ligation are summarised in Figure 2. The signalling subunits of the BCR are CD79A (Igα) and CD79B (Igβ). Both contain a single ITAM motif, which initiates signal transduction after activation (Dal Porto et al., 2004; Flaswinkel and Reth, 1994). ITAMs contain a consensus sequence surrounding two tyrosines which can be phosphorylated and then bind *src*-homology 2 (SH2) domains (Cambier, 1995; Reth, 1989). The exact

mechanism is not clear but it is thought that increased recruitment of *src*-family protein tyrosine kinases such as LYN, FYN, BLK and LCK following BCR microclustering leads to the phosphorylation of the ITAM tyrosines (Clark et al., 1992; Dal Porto et al., 2004; Pleiman et al., 1994). Phosphorylation of both tyrosines in an ITAM site creates a binding site for SYK. SYK then autophosphorylates itself and becomes active (Rowley et al., 1995). SYK then functions as a major hub in the activation of downstream signalling pathways (Mócsai et al., 2010). It has been shown to be an essential kinase (Turner et al., 1995) (Cheng et al., 1995) and is of major importance for B cell development (Turner et al., 1997). One of its targets that is phosphorylated and activated is B cell linker (BLNK), also known as SH2 domain–containing leukocyte protein of 65 kDa (SLP-65) or B cell adaptor containing SH2 domain (BASH). BLNK acts as a signalling scaffold and connects the BCR to various other signalling molecules including growth factor receptor bound protein 2 (GRB2), PLCγ and VAV proteins (Fu et al., 1998; Goitsuka et al., 1998; Wienands et al., 1998).

LYN also phosphorylates motifs on CD19 to recruit PI3K (Fujimoto et al., 2000; Tuveson et al., 1993). PI3K then phosphorylates the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). BTK binds PIP_3 via its pleckstrin homology domain and becomes activated (Saito et al., 2001). BTK is a kinase of major importance and mutations in BTK lead to X-linked immunodeficiency (Xid) in mice (Rawlings et al., 1993) and X-linked agammaglobulinaemia (XLA) in humans (Tsukada et al., 1993).

Apart from SYK and the *src*-family kinases, in particular LYN, BTK is the third critical upstream kinase in BCR signalling from which more diverse and distal signalling event emanate (Dal Porto et al., 2004).

Activation of BLNK by SYK leads recruitment of its signallosome to the plasma membrane including PLCγ2 that connects BCR cross-linking to Ca^{2+} influx (Fu et al., 1998; Goitsuka et al., 1998; Ishiai et al., 1999; Wienands et al., 1998). In addition, BLNK also interacts with BTK (Hashimoto et al., 1999) and phosphorylation by both SYK and BTK are required for optimal PLCγ2 activation (Chiu et al., 2002). In TCR signalling in T cells, recruitment of the BLNK homologue SH2 domain–containing leukocyte protein of 76 kDa (SLP-76) is mediated through the protein linker for activation of T cells (LAT) and the adaptor protein GRB2-related adaptor downstream of SHC (GADS) (Boerth et al., 2000; Ishiai et al., 2000; Zhang et al., 1998). Interestingly, expression of SLP-76 alone cannot rescue BLNK deficiency in B cells, only if LAT and GADS are co-expressed, BCR signalling is restored suggesting that BLNK and SLP-76 are recruited to the membrane by different mechanisms. Indeed, it was shown that BLNK is directly recruited to CD79A via a non-ITAM tyrosine residue (Engels et al., 2001; Kabak et al., 2002). Since LAT is critical in TCR signalling (Zhang et al., 1999), due to the essential recruitment mechanism of SLP-76 to the membrane, this might also explain the lack of requirement for the LAT homologue linker for activation of T cells 2 (LAT2) in B cells (also known as non-T cell activation linker (NTAL) or linker for activation of B cells (LAB) (Wang et al., 2005; Zhu et al., 2004). Given that

LAT2 can even substitute LAT in T cell development (Brdicka et al., 2002) its dispensability in B cell development might be due to BLNK being effectively recruited to the membrane by a different mechanism than SLP-76 in T cells, i.e. not relying on an adaptor for its localisation upon BCR stimulation. However, LAT2 might serve other functions in B cells.

Activated PLCγ2 catalyses the hydrolysis of PIP_2 to diacylglycerol (DAG) and inositol-3,4,5-phosphate (IP_3). IP_3 leads to the release of Ca^{2+} into the cytosol, mostly from the ER by binding to IP_3-receptors on the ER membrane (Sugawara et al., 1997). The Ca^{2+} decrease in the ER is sensed by stromal interaction molecules 1 (STIM1) and 2 (STIM2), which translocate to ER-plasma membrane junctions to open Ca^{2+} release-activated Ca^{2+} channels (CRAC) formed by ORAI proteins which pump Ca^{2+} from the extracellular space into the cytoplasm leading to a sustained Ca^{2+} release (Kurosaki et al., 2010; Shaw et al., 2013). Ca^{2+} in turn leads to the activation of calmodulin, which activates calcineurin phosphatase (CN) (Crabtree and Olson, 2002; Klee et al., 1998; Winslow et al., 2006). CN then initiates the translocation of transcription factor nuclear factor of activated T-cells (NFAT) into the nucleus by dephosphorylating N-terminal serines on NFAT (Crabtree and Olson, 2002). Calcium and DAG also activate protein kinase C (PKC) leading to NFκB signalling (Dal Porto et al., 2004; Dolmetsch et al., 1997; Saijo et al., 2002; Trushin et al., 1999).

In addition to these transcription factors, signalling by the mitogen-activated protein kinases (MAPK) p38 MAPK, ERK and c-Jun NH2-terminal kinase (JNK) can lead to activation of several transcription

factors. ERK activates ETS domain-containing protein 1 (ELK1) and myelocytomatosis oncogene (c-MYC), JNK activates jun proto-oncogene (c-JUN) and activating transcription factor 2 (ATF-2) and p38 MAPK activates ATF-2 and MAX (Dal Porto et al., 2004; Dong et al., 2002).

To activate MAPK signalling, several, partially redundant signalling pathways are found in B cells. DAG has been shown to activate the small GTPase resistance to audiogenic seizures (RAS) via its exchange factor RAS guanyl releasing protein (RASGRP). Activation of RAS results in signalling via the v-raf-leukemia viral oncogene 1 (RAF1) followed by activation of mitogen-activated protein kinase kinase kinase 1 (MEKK1 or MAP3K1) and leading to ERK activation (Dal Porto et al., 2004; Ehrhardt et al., 2004; Nagaoka et al., 2000; Oh-hora et al., 2003; Teixeira et al., 2003). Another minor mechanism for RAS activation is through BLNK-GRB2-Son of sevenless homologue 1 (SOS1). SOS1 is also an exchange factor for RAS (D'Ambrosio et al., 1996; Kurosaki et al., 2010; Nagai et al., 1995).

Activation of p38 MAPK and JNK can be achieved by a pathway involving VAV and RAS-related C3 botulinum substrate (RAC) proteins (Hashimoto et al., 1998; Ishiai et al., 1999). Both VAV family proteins (VAV1/VAV2/VAV3) and RAC proteins (RAC1/RAC2) display intrafamily redundancies but are essential for the generation of functional B cells as mice deficient in these proteins display defects in B cell development (Henderson et al., 2010; Tarakhovsky et al., 1995; Walmsley et al., 2003). VAV proteins are connected to the BCR via BLNK and GRB2 (Fu et al.,

1998; Johmura et al., 2003). In addition to activation of p38 and JNK, both RAC and VAV proteins are recruited to LAT2 for internalisation of the BCR (Malhotra et al., 2009). Moreover, VAV3 also activates PI3K via RAC1 and is indispensable for PI3K signalling and thus production of PIP_3 and calcium flux (Inabe et al., 2002).

1.4.2 CD40 receptor signalling

Co-stimulatory receptors can be divided into two families, the CD28/CD80 family and the TNF receptor (TNFR) family. CD40 is a member of the latter together with the other co-stimulatory receptors OX40, BAFFR, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B cell maturation antigen (BCMA) and receptor activator of nuclear factor κ B (RANK). The ligand for CD40 is CD40L also known as CD154, a TNF family ligand. Signals through CD40 are crucial second signals for B cells (Elgueta et al., 2009). Most of the CD40 signalling activity is mediated through the activation of the TNF-associated factors (TRAFs), a family of E3-ubiquitin ligases, and leads to the activation of NFκB, MAPKs, PLCγ and PI3K signalling pathways (Elgueta et al., 2009). Engagement of CD40 leads to its trimerisation but higher order structures can also be formed (Haswell et al., 2001; Pullen et al., 1999a). These complexes directly or indirectly recruit TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 to their cytoplasmic tails (Pullen et al., 1999b; Pullen et al., 1998).

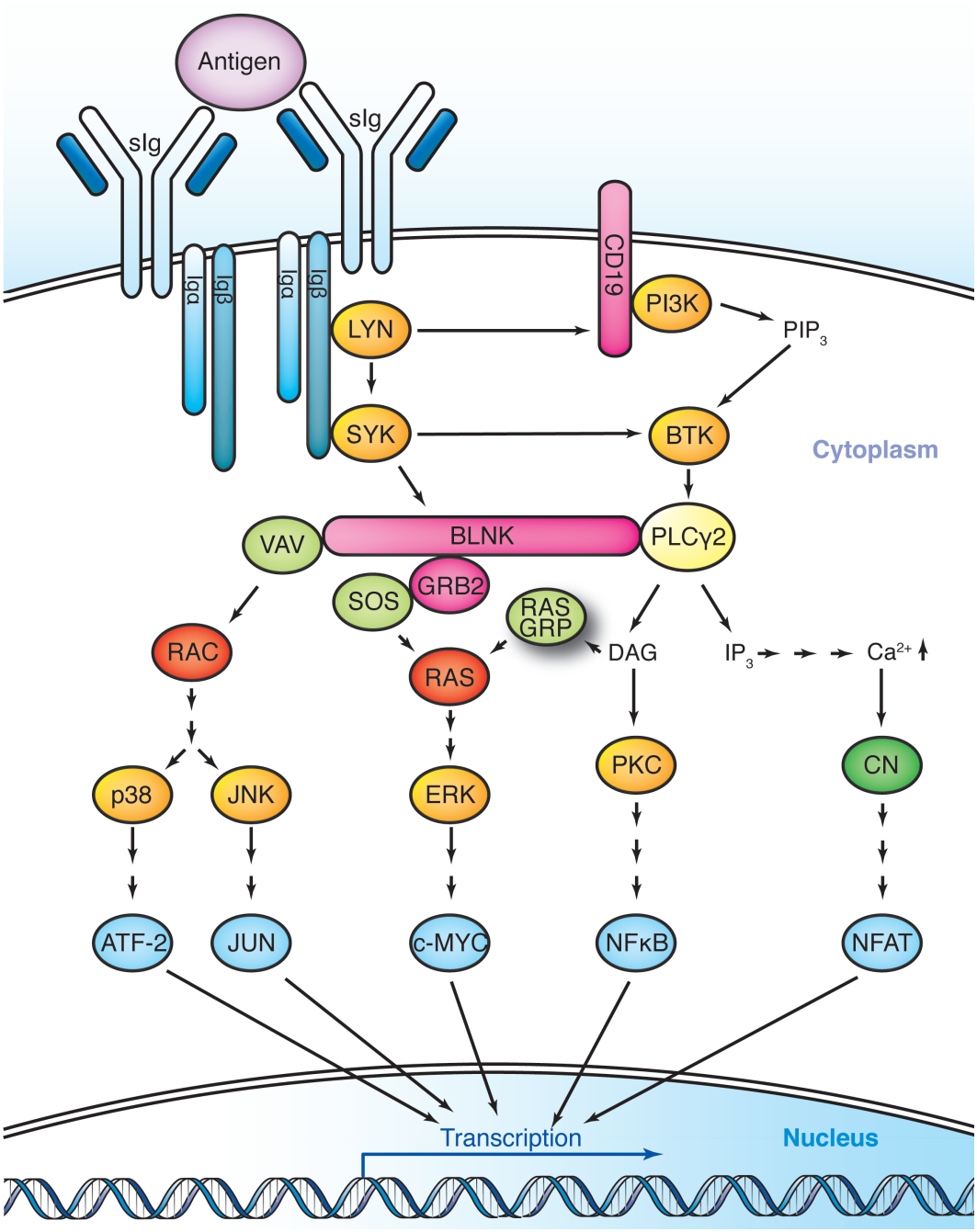

Figure 2: Simplified BCR signalling scheme.

Upon cognate antigen binding and cross-linking of surface immunoglobulin (sIg), the BCR recruits *src*-family kinases to the ITAMS of its Igα and Igβ subunits. Most prominently, kinase activity by LYN leads to phosphorylation of ITAMs and recruitment of SYK, which upon autophosphorylation gets activated to phosphorylate downstream signalling molecules such as BTK and BLNK. These in turn activate signalling via phospholipids and calcium or GTPases to activate MAP-

kinase signalling via p38, JNK and ERK. These pathways then lead to the translocation of transcription factors such as ATF-2, JUN, c-MYC, NFκB, or NFAT into the nucleus and initiation of transcription of target genes. Details in the text. Kinases are shown in orange, GTPases in red, guanine nucleotide exchange factors in light green, phosphatases in dark green, adaptors in pink and transcription factors at the bottom in light blue.

Recruitment of TRAF2 to CD40 in B cells leads to activation of JNK, p38 and thymoma viral proto-oncogene 1 (AKT) pathways (Lee et al., 1997). The activation of JNK and p38 and c-JUN is dependent on MAP3K1 (Gallagher et al., 2007).

The mechanism of activation of the NFκB pathway by TRAFs upon CD40 ligation has been elucidated recently (Gardam et al., 2008; Vallabhapurapu et al., 2008). A heterodimer of TRAF2 and TRAF3 in complex with cellular inhibitor of apoptosis 1 (cIAP1) and 2 (cIAP2) constantly degrades the NF-κB-inducing kinase (NIK), which would activate the non-canonical NFκB pathway. Upon CD40 engagement TRAF2 and TRAF3 are recruited to the receptor and degraded by autoubiquitinylation or cIAP1 and cIAP2-mediated degradation, thus preventing the degradation of NIK. NIK then accumulates though new protein synthesis and activates NFκB signalling (Brown et al., 2002; Elgueta et al., 2009; Hostager and Bishop, 2013; Vallabhapurapu et al., 2008; Zarnegar et al., 2008).

In B cells, casitas-B-lineage lymphoma protein-B (CBLB) has been shown to have a negative role in the regulation of CD40 signalling by reducing the recruitment of TRAF2 to CD40 leading to reduced NFκB and JNK activation (Qiao et al., 2007). Conversely, CBLB has the opposite role in DCs (Arron et al., 2001). Similarly, Janus kinase (JAK) signalling by JAK3 directly binding to CD40 and being critical for B cell gene expression (Hanissian and Geha, 1997) could not be verified in human B cells as JAK3 was not phosphorylated here. However, monocytes could

phosphorylate JAK3 upon CD40 ligation again showing differences in CD40 signalling between B cells and other cell types (Revy et al., 1999).

1.4.3 Interleukin-4 receptor signalling

IL-4 binds the IL-4 receptor (IL-4R) to initiate signalling. There are two types of IL-4 receptors: type 1 receptor is composed of IL-4Rα chain and the IL-2 receptor common γ chain (γ_c) whereas the type 2 receptor consists of the IL-4Rα chain and the IL-13α1 receptor chain, to both of which IL-4 can bind via the IL-4Rα chain and then recruit the second chain for signalling (Luzina et al., 2012). The related cytokine IL-13 can bind to type 2 receptors through binding to the IL-13Rα1 chain and initiate similar signalling mechanisms but with distinct outcomes depending on the expression to the types of receptor on different cell types and slightly different activation of downstream signalling pathways (Wills-Karp and Finkelman, 2008). In addition, IL-13 can bind to another chain, termed IL-13Rα2, mostly acting as a decoy receptor (Kawakami et al., 2001; Luzina et al., 2012). Signalling through IL-4 has been implicated with several different phenotypes in particular switching to IgE in B cells and polarisation of Th0 cells towards the Th2 lineage. IL-13 on the other hand is more associated with mediating airway hyperresponsiveness and mucus secretion in allergic reactions (Wills-Karp and Finkelman, 2008).

The IL-4R chains lack intrinsic kinase activity but are associated with JAKs. IL-4 signalling is initiated by receptor dimerisation allowing trans- and autophosphorylation of JAKs and tyrosines on the receptor chains

(Luzina et al., 2012). Whereas type 1 receptors activate JAK1 and JAK3 via JAK3-mediated binding to γ_c, the type 2 receptor activates JAK1, JAK2 and Tyk2 via IL-13Rα1 binding of JAK2 and Tyk2 (Wills-Karp and Finkelman, 2008). Moreover, SYK has been found to constitutively bind to IL4Rα1 in human neutrophils (Ennaciri and Girard, 2009). The phosphorylated tyrosines on the receptor chains serve as binding site for proteins with SH2-domains. Subsequent to JAK binding and phosphorylation of sites on the receptor chains, signal transducer and activator of transcription 6 (STAT6) can be recruited and phosphorylated by JAKs. This leads to dimerisation and translocation of STAT6 into the nucleus to act as a transcriptional activator (Luzina et al., 2012; Nelms et al., 1999; Wills-Karp and Finkelman, 2008; Witthuhn et al., 1994). Moreover, these residues allow binding of insulin receptor substrate (IRS) proteins, either IRS1 or IRS2, the type of which depending on the cell type with IRS2 expressed in haematopoietic cells and IRS1 in non-haematopoietic cells (Keegan et al., 1994; Luzina et al., 2012; Nelms et al., 1999; Wills-Karp and Finkelman, 2008). IRS proteins become phosphorylated and act as signalling scaffold leading to PI3K followed by AKT activation as well as signalling via GRB2 and SOS resulting in MAPK activation. (Nelms et al., 1999; Wills-Karp and Finkelman, 2008). Interestingly, another mechanism that seems to differentiate IL-13 from IL-4 signalling in macrophages is that activation of IRS-2 is only found by activation of type 1 receptors. Lack of type 1 receptors on IL-4 stimulated bone-marrow derived macrophages led to reduced mRNA expression of

alternatively activated macrophage marker genes such as *Arginase 1* (*Arg1*), found in inflammatory zone (*Fizz1*) and chitinase-like 3 (*Ym1*) (Heller et al., 2008).

1.4.4 BAFF and APRIL signalling

Like CD40L, BAFF (also known as TNFSF13B, BLyS or TALL-1) and APRIL (also known as TNFSF13 or TALL-2) belong to the TNF ligand family. Both BAFF and APRIL are homotrimeric type II transmembrane proteins (Mackay and Schneider, 2009). APRIL can also be trans-spliced with the neighbouring gene TNF-related weak inducer of apoptosis (TWEAK) which is gives rise to another ligand called TWE-PRIL about which little is known (Pradet‐Balade et al., 2002). Both BAFF and APRIL are proteolytically processed to form soluble ligands but BAFF is also found in a membrane bound form (Mackay and Schneider, 2009). BAFF usually forms trimers but can also form a 60-mer (Liu et al., 2003). BAFF and APRIL both bind to BCMA and TACI. Additionally, BAFF also binds and signals through BAFFR and was found to bind to the Nogo-66 receptor on neurons (Mackay and Schneider, 2009; Vincent et al., 2013; Zhang et al., 2009). APRIL can bind to heparin sulphate proteoglycans such as syndecans found on pre-B cells and plasma cells but does not signal through them. However, it is still biologically active when bound in this form (Huard et al., 2008; Kimberley et al., 2009). The binding affinities of the two ligands differ between the three receptors and allow distinct roles of each cytokine and receptor combination (Mackay and Schneider,

2009). Binding of a homotrimeric ligand induces signalling by trimerising the receptor although further multimerisation of the ligands might be required as in the case of BAFF or APRIL binding to TACI (Bossen et al., 2008; Kimberley et al., 2009).

Receptor oligomerisation leads to recruitment of TRAFs and downstream non-canonical NFκB signalling, again similar to CD40 signalling. BAFFR recruits TRAF3 whereas TACI recruits TRAF2 and TRAF6 (Baud et al., 1999; Gardam et al., 2008; Mackay and Schneider, 2009; Xie et al., 2007; Xu and Shu, 2002).

The signalling mechanism used by BAFFR to activate non-canonical NFκB is so far identical to the one used by CD40. NIK kinase activates non-canonical NFκB signalling but is normally degraded by a complex of TRAF2 and TRAF3 in complex with cIAP1 and cIAP2. Upon BAFFR ligation, TRAF3 is recruited to the receptor at the plasma membrane and the complex is unable to degrade NIK, leading to NIK accumulation and activation of the non-canonical NFκB signalling cascade (Vallabhapurapu et al., 2008). Other signalling pathways are also engaged by BAFFR but the exact signalling mechanisms of their activation and interaction partners are so far obscure. These include weak activation of canonical NFκB and activation of PI3K (Enzler et al., 2006; Patke et al., 2006). BAFFR ligation leads to Igα and Syk phosphorylation, which activates PI3K and ERK signalling (Schweighoffer et al., 2013). PI3K leads to AKT1 activation via phosphoinositide-dependent kinase 1 (PDK1). Additionally, PKCβ is also involved in the AKT1 activation (Patke et al., 2006). Together with signals

from the kinase PIM2, which is activated by BAFF via the non-canonical NFκB pathway, BAFF-induced AKT1 activation results in increased metabolic fitness and production of anti-apoptotic proteins, in particular MCL1, supporting B cell survival (Enzler et al., 2006; Mackay and Schneider, 2009; Patke et al., 2006; Woodland et al., 2008).

1.4.5 Toll-like receptor signalling

Signalling pathways for TLRs have mostly been elucidated in myeloid cells such as DCs and macrophages (Peng, 2005). The major TLR signalling pathways in these cells have been reviewed extensively and are summarised in Figure 3 (Broz and Monack, 2013; Kawai and Akira, 2009; Kawai and Akira, 2011; Kondo et al., 2012). The extent to which they are conserved in B cells and between mouse and human are not completely clear at the moment. Moreover, TLR expression is B cell subset specific and differs between mouse and human. In particular, TLR4 seems to be present at very low levels on human B cells whereas it is readily detectable on mouse B cells (Bekeredjian-Ding and Jego, 2009; Bekeredjian-Ding et al., 2005; Hornung et al., 2002). It should be noted that for example the roles of TRAF proteins seem to be different in B cells and myeloid cells. For example, whereas TRAF3 leads to enhanced responses in macrophages it seems to be a negative regulator in TLR responses by B cells (Hacker et al., 2006; Xie et al., 2011).

Models for TLR signalling pathways in B cells have been proposed, however these are mainly based on correlations with myeloid cells and

have mostly not been directly tested (Peng, 2005). TLR7 and TLR9 are generally strongly expressed in B cells and have been proposed to lead to activation of the IL-1 receptor-associated kinases (IRAKs). IRAKs in turn activate TRAF6 leading to activation of TGFβ-activated kinase (TAK1). This pathway then leads to MAPK and IKK activation resulting in activator protein-1 (AP-1)-family and NFκB-mediated transcription of target genes (Peng, 2005). The proposed model for TLR4 activation through myeloid differentiation primary response gene 88 (MyD88) leads to the activation of the same signalling pathways as proposed for TLR7 and TLR9. In addition, the TLR4 pathway through TIR-domain-containing adaptor protein inducing IFNβ (TRIF) leads to activation of TRAF family member-associated NFκB activator (TANK)-binding kinase 1 (TBK1) and IRF3 mediated transcription, atypical IKK activation and NFκB-mediated transcription and activation of protein kinase R (Peng, 2005).

Some roles for TLR signalling in B cells have been mentioned throughout the text but several others have been described (Bekeredjian-Ding and Jego, 2009; Browne, 2012; Hua and Hou, 2013; Rawlings et al., 2012). Generally, TLRs in B cells are involved in the development of B cells, B cell cytokine secretion, CSR and antibody responses (Barr et al., 2007; Hayashi et al., 2005; Hou et al., 2008; Hou et al., 2011; Lutzker et al., 1988). In particular, autoantibody production is regulated by TLRs as TLR ligands such as DNA, contained in immune complexes, can be a BCR ligand at the same time as a TLR ligand and lead to B cell activation by

delivering the first and second signal simultaneously, similar to type I T-independent antigens (Leadbetter et al., 2002; Vinuesa and Chang, 2013).

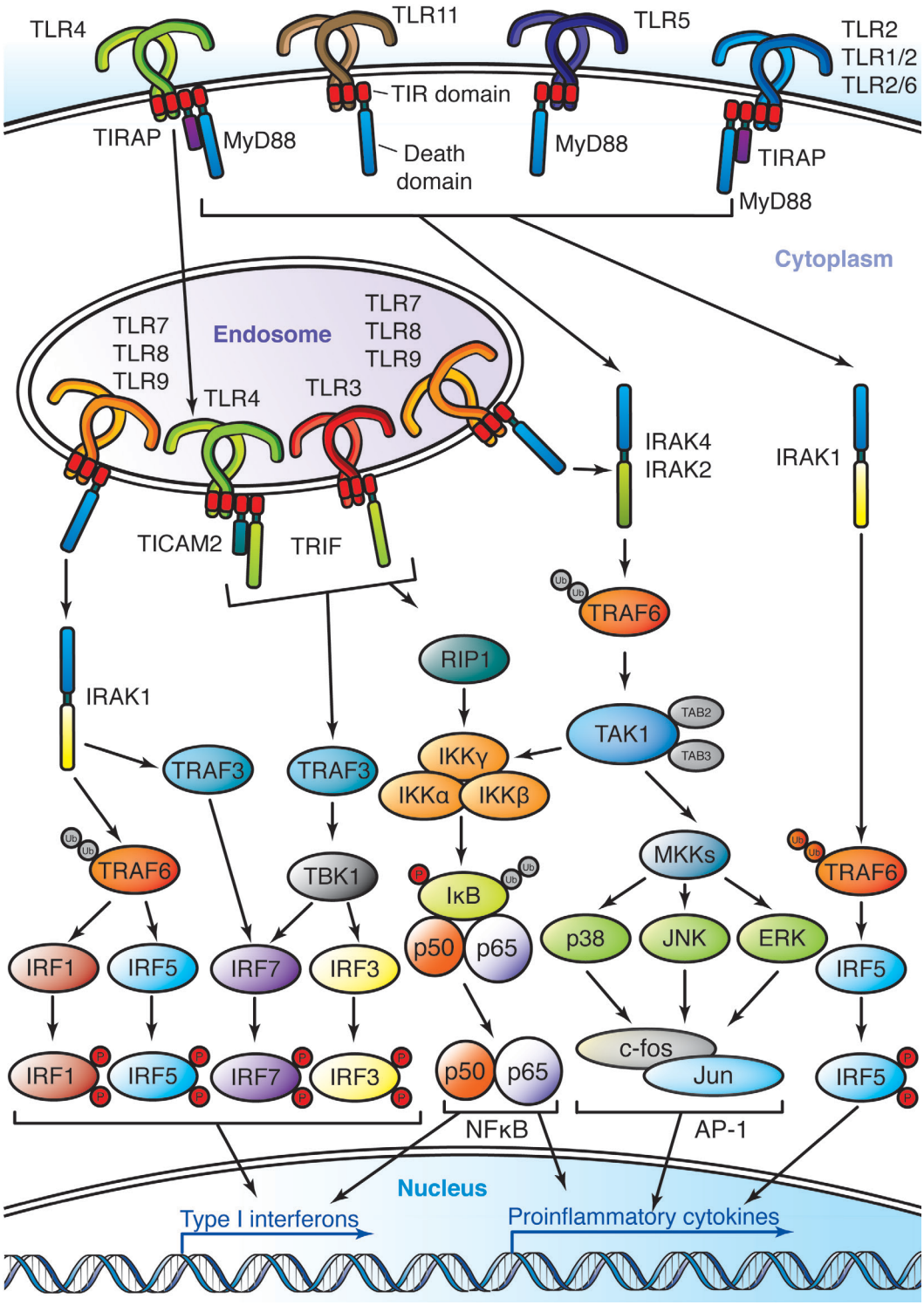

Figure 3: Simplified TLR signalling scheme derived from myeloid cells.

Cell surface TLRs signal via the adaptor protein MYD88 to IRAKs. TLR3 signals from the endosome using the adaptor TRIF. TLR4 signals first from the cell surface via MYD88 and after endocytosis via TRIF using the adaptors TIRAP and TICAM2 respectively. IRAKs phosphorylate and

activate TRAFs, a family of ubiquitin ligases, which can activate signalling by NFκB, IRFs and MAP kinases. IRFs generally lead to the transcription of type 1 interferon genes although they can also drive proinflammatory cytokine expression depending on the cellular context. TRAF6 though autoubiquitinylation recruits TAK1 via TAB adaptor proteins. TAK1 activates the IKK complex through phosphorylation which leads to lysosomal degradation of IκB and thus release and translocation of p50 and p65 NFκB subunits into the nucleus driving expression of both proinflammatory and type 1 interferon genes. In addition, TAK1 activates the MAP kinase cascade leading to phosphorylation and activation of p38, JNK and ERK resulting in translocation of transcription factor AP-1 into the nucleus and consequently transcription of proinflammatory genes. Most of these signalling pathways were elucidated in macrophages or dendritic cells and may differ in B cells. TIRAP, TIR domain-containing adaptor protein; TICAM2, toll-like receptor adaptor molecule 2; IkB, Inhibitor of kB; RIP1, Receptor-interacting serine/threonine-protein kinase 1; TIR domain, Toll/IL-1 receptor homology domain; Ub, Ubiquitin; other abbreviations are found in the main text or the list of abbreviations.

1.5 The Themis family

The thymocyte-expressed molecule involved in selection (Themis) family is a recently identified group of genes with two to three paralogues among mammals. The family is characterised by the presence of a cysteine-containing, all-β in Themis (CABIT) domain, which is predicted to be globular, consisting of extended β-sheets that contain a conserved cysteine residue. This domain is often found together with a highly conserved proline-rich region and in the case of several higher evolved species it has duplicated within the same protein, giving rise to two consecutive CABIT domains. During evolution, the motif has been conserved, being found in several metazoan genomes down to cnidarians (Johnson et al., 2009).

The function of the CABIT domain is still elusive and whether it purely serves a structural function or has an enzymatic activity is currently unknown although recent results suggest that the conserved cysteine in THEMIS, termed THEMIS1 from here on for clarity, is dispensable for T cell development (Zvezdova et al., 2014). The conserved cysteine residue is predicted to be on the surface of THEMIS1 and such residues without adjacent acidic or histidine residues have been observed in E2 ubiquitin ligases, thiol redox and sulphur carrier proteins. It is therefore possible that the CABIT domain has a biochemical function (Johnson et al., 2009).

In mice there are five CABIT domain-containing proteins: Grb2 associated, regulator of ERK/MAPK1 (GAREM also known as Fam59A) and GAREM-like (GAREML also known as GAREM2 or Fam59b) as well as the three

Themis family members: THEMIS1, (also known as GASP, C6orf190 in humans), THEMIS2 (also known as ICB-1, ICB1, BC013712 in mouse or C1orf38 in humans) and THEMIS3 (*9130404H23Rik,* in mouse). In contrast to mice, humans lack THEMIS3. Whereas GAREM and GAREML only contain one N-terminal CABIT domain, a proline-rich domain and a C-terminal sterile α-motif (SAM) domain (Johnson et al., 2009; Taniguchi et al., 2013; Tashiro et al., 2009), the Themis family bears two consecutive CABIT domains followed by a proline-rich region (Johnson et al., 2009). The structure of murine Themis2 is outlined in Figure 4.

Among the Themis family proteins only THEMIS1 and THEMIS2 have been studied at the protein level so far. Evidence for THEMIS3 stems either from sequence predictions, large-scale transcriptomic or proteomic approaches (Lattin et al., 2008; Su et al., 2004; Villén et al., 2007; Wu et al., 2009).

1.5.1 Themis1

A function for the first family member, *Themis1*, has been discovered in T cells (Fu et al., 2009; Johnson et al., 2009; Kakugawa et al., 2009; Lesourne et al., 2009; Patrick et al., 2009). *Themis1*-deficient mice show a block during positive selection of T cells, which results in decreased numbers of single-positive thymocytes. Two groups also reported a defect in negative selection using either a mouse mammary tumour virus-based superantigen model (Fu et al., 2009) or a H-Y transgenic TCR model

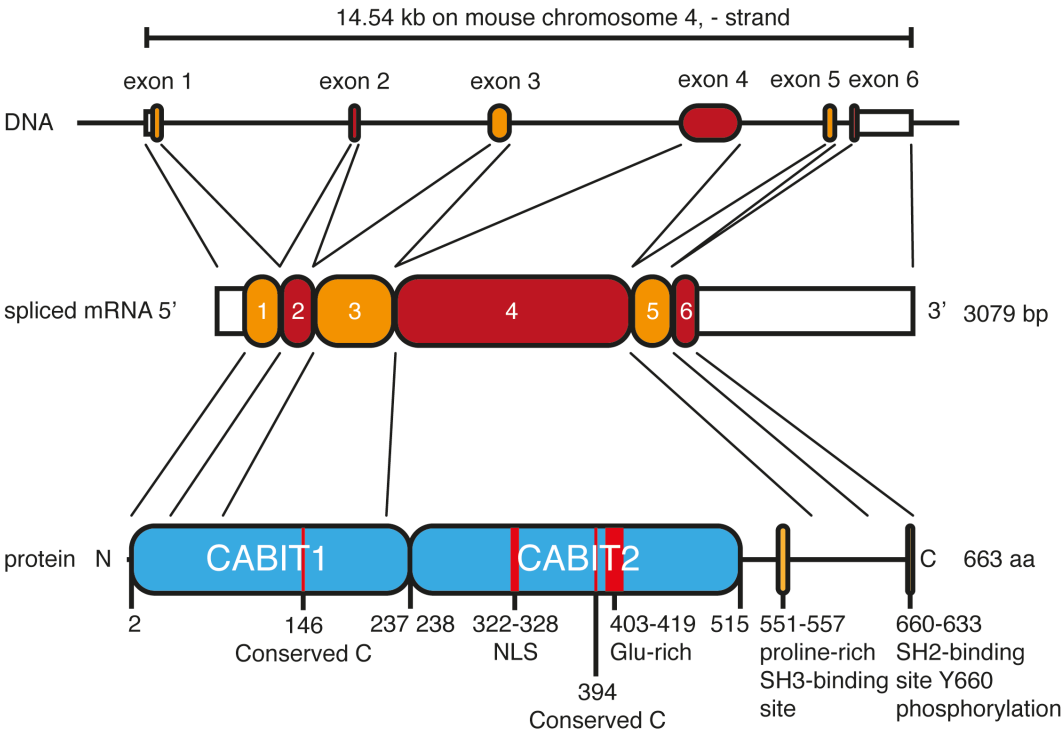

Figure 4: Gene and protein structure of murine *Themis2*.

Themis2 is encoded by six exons located on mouse chromosome 4. All six exons are spliced into one mRNA which is translated into a protein consisting of two N-terminal, consecutive CABIT domains containing conserved cysteine residues and a nuclear localisation sequence (NLS) as well as a glutamate-rich stretch. The C-terminus contains a proline-rich region comprising an SH3-binding site and further downstream a tyrosine residue (Y660), which upon phosphorylation, has been shown to be an SH2-binding site. Exon 4 is the biggest exon of *Themis2* and encodes the C-terminal part of the CABIT1 domain, the whole CABIT2 domain and the SH3-binding site. It is also the only exon in *Themis2* encoding a phase shift. Thus, following deletion of exon 4, exon 5 is out of frame with respect to exon 3.

(Lesourne et al., 2009). However, the results were not completely consistent since another study using the same two models saw no effect on negative selection (Johnson et al., 2009). This might be due to the way THEMIS1 is thought to affect the selection process. It was recently reported that THEMIS1 functions as a rheostat that sets the threshold for positive and negative selection. In the absence of THEMIS1, which lowers the selection threshold, signals that would usually be interpreted as positive selection signals cross the threshold and cells are negatively selected instead of positively selected resulting in diminished single-positive thymocyte numbers. Hence, positive selecting TCR interactions in wild type cells are interpreted as strong self-reactive interactions in *Themis1*-deficient cells and are consequently deleted from the repertoire (Fu et al., 2013). In addition, the differences observed in the initial studies could be due to the experimental approach or due to the mice used since the first two studies used *Themis1*-deficient mice targeted at exon 1 (Fu et al., 2009; Lesourne et al., 2009) and the latter study used only a point mutated mouse strain leading to a mutant THEMIS1 truncated at the end of the second CABIT domain which might have sustained this function in negative selection (Johnson et al., 2009).

The role of THEMIS1 in positive selection also coincides with its highest expression in late DN and in DP thymocytes. However, it is still detectable in more mature T cells and its expression is not dependent on TCR signalling (Fu et al., 2009; Johnson et al., 2009; Lesourne et al., 2009; Patrick et al., 2009). The activation and proliferation of *Themis1*-deficient

peripheral T cells seems to be reduced although this still awaits confirmation using conditional ablation of *Themis1* since this might be a defect carried through from development (Brockmeyer et al., 2011; Patrick et al., 2009). Moreover, this defect was not found in all studies (Lesourne et al., 2009).

The three initial studies came to different conclusions concerning the involvement of THEMIS1 in proximal TCR signalling (Fu et al., 2009; Johnson et al., 2009; Lesourne et al., 2009). However, it has become clear that THEMIS1 is part of the LAT signallosome and that recruitment of THEMIS1 to the LAT signallosome is required for its function (Fu et al., 2013; Lesourne et al., 2012; Paster et al., 2013). It was shown that THEMIS1 is tyrosine phosphorylated after TCR cross-linking with similar kinetics to the TCR signalling scaffold proteins LAT and SLP-76 (Brockmeyer et al., 2011; Fu et al., 2009; Lesourne et al., 2009; Paster et al., 2013). Overexpression studies of the kinases LCK and ZAP70 indicate that they are responsible for THEMIS1 phosphorylation (Paster et al., 2013). *In vitro*, phosphorylation of THEMIS1 was dependent on SLP-76, LCK and LAT (Brockmeyer et al., 2011). Some studies failed to detect more distal TCR signalling defects (Johnson et al., 2009; Lesourne et al., 2009; Patrick et al., 2009) whereas others have reported small defects in ERK and NFAT/AP-1 signalling and Ca^{2+} influx which seem to depend on the strength and nature of the stimulus (Brockmeyer et al., 2011; Fu et al., 2013; Fu et al., 2009).

Reported interactors also fit into the role of THEMIS1 as a signalling

scaffold in TCR signalling cascade including the adaptor GRB2 (Brockmeyer et al., 2011; Johnson et al., 2009; Lesourne et al., 2009; Patrick et al., 2009), the IL-2 inducible T cell kinase (ITK) (Fu et al., 2009), PLCγ1 (Brockmeyer et al., 2011; Fu et al., 2009; Zvezdova et al., 2014) and VAV1 (Lesourne et al., 2012). GRB2 interacts via its N-terminal *src*-homology 3 (SH3) domain with THEMIS1 (Patrick et al., 2009). This interaction was dependent on the proline-rich region of THEMIS1 (Lesourne et al., 2012; Okada et al., 2014; Zvezdova et al., 2014) however one study concluded that it was independent of it (Patrick et al., 2009). Interestingly, SOS does not seem to interact with THEMIS1 (Lesourne et al., 2012) which might be due to it using the same SH3 domain on GRB2 and their binding to GRB2 therefore being mutually exclusive (Cussac et al., 1994; Patrick et al., 2009). GRB2 also links THEMIS1 to LAT (Brockmeyer et al., 2011; Paster et al., 2013). The interaction of THEMIS1 with GRB2 seems to be constitutive and slightly increased upon TCR stimulation (Brockmeyer et al., 2011; Lesourne et al., 2009) but again this has not been found in all studies (Patrick et al., 2009). GRB2 has also recently been shown to have an important role in positive selection via conditional deletion (Jang et al., 2010). The similar results found in these papers indicate that the interaction of THEMIS1 with GRB2 together might be crucial for thymic selection and cell fate decision (Gascoigne and Palmer, 2011). Since there is an array of proteins in T cells containing SH2 domains, it has been speculated that phosphorylated THEMIS1 might interact with SH2 domains of other proteins (Allen, 2009).

In terms of protein localisation, THEMIS1 has been detected in the cytoplasm and in the nucleus. In two studies transfection of tagged THEMIS1 into T cell lines showed exclusion form the nucleus (Johnson et al., 2009; Patrick et al., 2009). However, other studies located it to the cytoplasm and the nucleus, for example via subcellular fractionation and immunoblot. This is achieved via a putative nuclear localisation signal found in its sequence between residue 330 and 346 which was required for nuclear localisation (Fu et al., 2009; Lesourne et al., 2009; Lesourne et al., 2012; Okada et al., 2014; Zvezdova et al., 2014). The nuclear localisation of THEMIS1 has furthermore been confirmed by a study identifying it as a part of the human T-cell nucleolus proteome (Jarboui et al., 2011).

Recently, the functional domains of THEMIS1 were also tested for their contributions to THEMIS1 functions. Deletion of the NLS, the proline-rich SH3 binding site (PRS) or the core motifs of the two CABIT domains resulted in impaired phosphorylation as well as impaired GRB2-binding and the mutants could not restore T cell development in a *Themis1*-deficient background. Nuclear localisation was mediated by the core of CABIT1 and the NLS and both core mutants showed dominant-negative effects on T cell development (Okada et al., 2014). In another study the same requirements were found for the NLS and the proline-rich region, however, the CABIT domains were not required for THEMIS1 function in this study (Zvezdova et al., 2014). These results might be explained by the different mutations introduced into the CABIT domains. The former study

deleted CABIT core sequences of 24 to 25 amino acids length whereas the latter only introduced alanine point mutations of the conserved cysteines of CABIT1 and CABIT2, which could potentially be insufficient to abolish THEMIS1 CABIT domain functions.

Mutations in *Themis1* have also been associated with other phenotypes. The description of its phenotype led to a reinterpretation of the T-helper immunodeficiency of the LEC rat whose defect had hitherto been ascribed to the neighbouring gene *Ptprk*. However, upon re-examination it was found to be attributed to the rat orthologue of *Themis1* (Iwata et al., 2010). A more recent study identified another mutation of *Themis1* in a Brown-Norway rat colony. Reduced $CD4^+$ T cell levels were observed in these rats and the remaining T cells were skewed towards Th2 and Th17 phenotypes. The T cell lymphopenia originated in the thymus and was T cell intrinsic and lead to a high incidence of inflammatory bowel disease (IBD). The increase in IBD was partly due to defective suppressive activity of regulatory T cells (Chabod et al., 2012). Furthermore, it was reported that histone deacetylase 1 and 2 regulate the expression of THEMIS1 (Dovey et al., 2013). *THEMIS1* has also been found to be associated with celiac disease, another intestinal immune-mediated disease, in a genome-wide association study (Dubois et al., 2010) which was subsequently verified by two other studies (Bondar et al., 2013; Senapati et al., 2014). Another study found *Themis1* upregulated in thymocytes of prediabetic mice linking it to type 1 diabetes (Fornari et al., 2011). Interestingly, an intronic single nucleotide polymorphism (SNP) in *THEMIS1* has been

linked to several personality traits (Wang et al., 2012) and the possibility has been discussed that *THEMIS1* is associated with breast cancer (Bhatti et al., 2010).

1.5.2 Themis2

Less is known about the second member of the Themis family. *THEMIS2* was initially identified as “induced by contact to basement membrane-1” (ICB-1) in cultured, human cancer cells. Its transcript is upregulated upon binding of endothelial adenocarcinoma cells to Matrigel (Treeck et al., 1998). Later, *THEMIS2* was shown to be regulated under several different conditions.

Firstly, it became associated with the monocytic differentiation of acute myeloid leukaemia cell line HL-60 following stimulation with retinoic acid, vitamin D3 and dimethyl sulfoxide (DMSO), a model for myeloid differentiation, in which it was upregulated (Treeck et al., 2002). As predicted by the HL-60 model, in another study *THEMIS2* was undetectable in undifferentiated bone marrow but was induced in both lymphocyte-enriched and macrophage-enriched fractions of *in vitro* differentiated bone marrow cells cultured with macrophage-colony-stimulating factor (M-CSF) (Peirce et al., 2010). The previous study also reported four possible human splice variants termed *ICB-1-*α*, -*β*,* γ, and -δ (Treeck et al., 2002). Furthermore, *THEMIS2* was upregulated following IFNγ treatment of various cancer cell lines and its knockdown resulted in a stronger inhibition of proliferation by IFNγ (Treeck et al., 2005). In RAW

macrophages stimulation with the proinflammatory stimulus IFNγ also led to an increase in THEMIS2 levels whereas anti-inflammatory stimuli such as TGFβ and dexamethasone reduced THEMIS2 expression (Peirce et al., 2010). *In vivo* inflammatory conditions such as collagen-induced arthritis and influenza infection lead to THEMIS2 downregulation in splenocytes (Peirce et al., 2010). Conversely, it was found to be upregulated in human bronchial epithelial cells upon infection with rhinovirus or stimulation with platelet-activating factor (Katz et al., 2006).

The discovery of a putative, imperfect oestrogen response element (ERE) in the promoter region of *Themis2*, prompted the stimulation of an array of cancer cells with various oestrogen receptor-α (ERα) stimuli which were found to induce *Themis2* expression. However, the increase is slow and requires protein synthesis and is thus not a direct effect of ERα binding to the ERE (Bollmann et al., 2008). Conversely, RNA interference (RNAi) of *THEMIS2* enhances proliferation of various cancer cell lines in response to oestrogen. Higher levels of ERα-dependent genes like c-FOS are observed and more ERα is produced upon *THEMIS2* knockdown (Konwisorz et al., 2010). Additionally, silencing of *THEMIS2* reduces the vitamin D3 or all-trans retinoic acid-induced upregulation of E-cadherin, lactoferrin and ERβ in a breast cancer cell line and *THEMIS2* levels correlate with E-Cadherin levels in breast cancer samples (Haselberger et al., 2011). Knockdown of *THEMIS2* in a human breast cancer cell line and analysis of global gene expression suggest a network of oncogenic and TNF-related genes to be differentially regulated by THEMIS2. Silencing of

THEMIS2 in the human breast cancer cell line also leads to reduced responses to apoptosis inducing drugs (Treeck et al., 2012). Similar results were obtained with ovarian cancer cell lines. Knockdown of *THEMIS2* in these cells revealed oestrogen-responsive and again TNF related genes to be regulated by THEMIS2. Moreover, *THEMIS2* knockdown results in better cell proliferation of the analysed ovarian cancer cell lines (Treeck et al., 2013). Other links to both breast and ovarian cancer were found in genetic association studies. The G allele of an intronic single nucleotide polymorphism (SNP) in *THEMIS2* (A to G, rs1467465) is associated with lower breast cancer incidence in healthy individuals suggesting a protective role of this variation (Springwald et al., 2009). The same SNP is also associated with susceptibility to ovarian cancer with higher incidence of the A allele in ovarian cancer patients (Schüler et al., 2014).

Several other studies reported regulation of *Themis2* under different conditions. *Themis2* regulation was found in extrathymic Aire-expressing cells (eTACs) and its expression is repressed in these cells by autoimmune regulator (Aire) (Gardner et al., 2008). *Themis2* is also upregulated in pancreatic islet cells after IFNα treatment (Hultcrantz et al., 2007). In endometrial cancers, Ki-67 as well as *THEMIS2* expression is elevated compared to normal endometrium and *THEMIS2* positively correlates with v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (*HER2*) expression (Springwald et al., 2010).

Interestingly, HER2 also signals using GRB2 (Olayioye, 2001). As in the

case of THEMIS1, THEMIS2 has also been found to interact with GRB2 in macrophages. As for THEMIS1, this interaction seems to depend on the presence of the conserved proline-rich region (Peirce et al., 2010). The proline-rich region is a potential SH3-binding domain with an atypical sequence, which has been shown in other proteins to bind SH3 domains (Lock et al., 2000). Furthermore, THEMIS2 is tyrosine phosphorylated upon LPS stimulation and associates with LYN. This phosphorylation was identified to be on residue Y660, making its sequence a potential SH2-binding site at the C-terminal end of the protein. This residue was also required for association with LYN. Moreover, THEMIS2 was found to interact with VAV1, however neither the SH3- nor the SH2-binding site are required for this association. Overexpression of THEMIS2 in RAW macrophages leads to a slight increase in p38 and ERK phosphorylation but JNK signalling, NF-kB p65 and IRF3 translocation to the nucleus are unaffected. Furthermore, the overexpression leads to a twofold increase in TNFα production upon LPS stimulation but not upon stimulation with other TLR ligands such as PAM3 or poly-IC. IL-6 production and cyclooxygenase 2 (COX2) production are unaffected in this experimental system. This increase of TNFα production is dependent on both the SH3- and the SH2-binding sites and a 20 – 50 % knockdown of *THEMIS2* in human macrophages results in a 50 % reduction in TNFα release. The effect of *THEMIS2* on TNFα production is at least partially attributable to the transcriptional control of TNFα production (Peirce et al., 2010). Lastly, it was shown that THEMIS2 is phosphorylated shortly after BCR cross-

linking in primary mouse B cells, possibly by *src*-family kinases, suggesting that it might have a function in B cells (Lesourne et al., 2012).

1.5.3 *Garem* and *Garem*l

More recently studies on these two CABIT-domain containing proteins have been undertaken. GAREM was found to transduce signals from the epidermal growth factor (EGF) receptor and influence transformation activity of cells. Much like the Themis proteins it is tyrosine phosphorylated after receptor activation and binds to GRB2 in a phosphorylation and proline-rich region dependent manner. SH2 domain-containing phosphatase 2 (SHP2) binds to GAREM and regulates ERK signalling independent of SOS or GRB2-associated binder (GAB1) (Tashiro et al., 2009).

GAREML also mediates EGF signals but whereas GAREM is expressed ubiquitiously, GAREML is specifically expressed in the mouse, rat and human brain. Like GAREM, GAREML also is phosphorylated after receptor activation, binds to GRB2 and regulates ERK signalling via SHP2. Moreover GAREML responds to stimulation by insulin-like growth factor 1 inducing neuronal differentiation in a neuroblastoma cell line. Whereas GAREM is recruited to the nucleus GAREML is cytosolic. This might be due to an N-terminal NLS resembling a 14-3-3ε binding site in GAREM (Taniguchi et al., 2013).

1.5.4 Differences and similarities between *Themis1* and *Themis2*

In terms of overall structure, *Themis1* and *Themis2* are highly similar. Features of murine THEMIS2 are highlighted in Figure 4. Both feature two consecutive CABIT domains at the N-terminus followed by a PRS and a possible phosphorylation site at the C-terminal end. THEMIS2 is slightly longer with 663 amino acid in the mouse compared to 636 residues in THEMIS1. However, THEMIS1 and THEMIS2 amino acid sequences are highly similar (EMBOSS Needle: 31 % identical, 50 % similar in mouse) and are well conserved between mouse and human (65 % identical, 77 % similar for THEMIS2). Both also have a nuclear localisation sequence (NLS) found in the second CABIT domain and for both THEMIS1 and THEMIS2 it has been shown that they are found in the cytoplasm as well as in the nucleus (Fu et al., 2009; Jarboui et al., 2011; Lesourne et al., 2009; Lesourne et al., 2012; Okada et al., 2014; Zvezdova et al., 2014).

The proteins are so similar that THEMIS2 can substitute THEMIS1 and restore T cell development to wild type levels in *Themis1*-deficient mice if THEMIS2 is ectopically expressed in the T cell lineage. A construct with *Themis2* under the control of the human CD2 promoter was used to express THEMIS2 in T lineage cells. The construct was introduced retrovirally into the bone marrow of *Themis1*-deficient mice and showed complete rescue of T cell development (Lesourne et al., 2012).

In general, the currently known interactome of both proteins consists of proteins associated with T cell receptor (TCR) or B cell receptor (BCR)

signalling and both share GRB2 as a binding partner through the proline-rich domain (Patrick et al., 2009; Peirce et al., 2010).

The biggest difference observed so far is the tissue expression pattern. *Themis1* expression is restricted to T cells (Fu et al., 2009; Johnson et al., 2009; Lesourne et al., 2009) whereas according to microarray data, *Themis2* is expressed in B cells, macrophages and dendritic cells. *Themis3* has been detected in the intestine according to publicly available data (www.biogps.org and www.immgen.org) (Lattin et al., 2008; Su et al., 2004; Wu et al., 2009). Recently, public data from the phenotyping pipeline at the Wellcome Trust Sanger Institute reported a mouse containing a reporter construct in the *Themis2* locus and showed that *Themis2* is only expressed in tissues containing B cells, macrophages and dendritic cells (www.sanger.ac.uk/mouseportal) (Dyke and Hubbard, 2011). Furthermore chromatin-immunoprecipitation experiments from the ENCODE project (genome.ucsc.edu/encode) indicate that transcription factors typically associated with B cell development such as PAX5, EBF1 and PU.1 bind to the promoter region of human *THEMIS2* (Rosenbloom et al., 2013). Taken together, these results suggest tissue specific functions for each member of the Themis family.

1.6 Aims and hypothesis

It has been noted for a long time that there are strong similarities between BCR and TCR signalling pathways (Weiss and Littman, 1994). Often one protein will exert a certain function in T cells while its paralogue performs a similar function in B cells. Examples are the roles of SYK-family kinases ZAP70 and SYK (Mócsai et al., 2010), isoforms of PI3Ks (Okkenhaug, 2013), the adaptor proteins SLP-76 and BLNK (Koretzky et al., 2006) or the different requirements for the Rho-family guanine-nucleotide exchange factors VAV1/2/3 in T and B cells (Swat and Fujikawa, 2005). Given the important role for THEMIS1 in T cell development and the high degree of similarity between THEMIS1 and THEMIS2, I tested the hypothesis that THEMIS2 might have a function in B cell development or activation similar to that of THEMIS1 in T cells.

Therefore I measured the expression of the Themis-family in B lineage cells and show that *Themis2* is expressed throughout the B cell lineage in a non-redundant manner. After B cell activation *Themis2* is downregulated but is also not substituted by other CABIT domain-containing proteins. The creation of a *Themis2*-deficient mouse strain allowed the me to analyse the effects of loss of THEMIS2 in B cells but surprisingly I found that B cell development, activation and antibody responses are unaffected by a deficiency of THEMIS2.

2 Materials and methods

2.1 Mice

All mice were kept under specific pathogen-free conditions at the animal facility of the Medical Research Council National Institute for Medical Research (NIMR), and all animal experiments were treated according to institutional guidelines (NIMR Ethical Review Panel) and UK Home Office regulations. The C57BL/6N embryonic stem cell line JM8.F6 with the targeted allele Themis2$^{tm1a(KOMP)Wtsi}$ were provided by the Welcome Trust Sanger Institute and used to generate mice on a C57BL/6 background at the Hammersmith Hospital. Those mice were kindly provided by Matthew Peirce. In order to obtain the Themis2$^{tm1d(KOMP)Wtsi}$ allele, here further called Themis2^{KO}, Themis2$^{tm1a(KOMP)Wtsi}$ mice were crossed first to C57BL/6J-Tg(Prm-cre)70Og mice carrying the Cre recombinase under the control of the Protamine promoter (also known as PC3::Cre) (O'Gorman et al., 1997) and then to C57BL/6J-Tg(ACTFLPe)9205Dym mice with recombinase FLPe under the control of the human ACTB promoter (also known as ACTB::FLPe) (Rodríguez et al., 2000). All experiments used age and sex-matched littermate control animals.

B6.SJL-Ptprc^{a} (CD45.1^{+}) (Shen et al., 1985), Rag1^{tm1Mom} (Rag1-deficient) (Mombaerts et al., 1992) and Tg(TcraTcrb)425Cbn (OT-II) (Barnden et al., 1998) Il10^{tm1Flv} (IL-10 GFP reporter tiger mice) (Kamanaka et al., 2006), all on a C57BL/6 background, were obtained from the breeding facility at NIMR.

2.2 Mixed bone marrow chimeras

Bone marrow from femora and tibiae of B6.SJL-Ptprc^{a} and *Themis2*$^{KO/KO}$ and wild type littermate control donor mice was harvested separately. Red blood cells were lysed by resuspension in ACK lysis buffer for 2 min at room temperature. Cell concentrations were determined and B6.SJL-Ptprc^{a} bone marrow was mixed at the indicated ratios with either *Themis2*$^{KO/KO}$ or wild type control bone marrow and then intravenously injected into recipient Rag1-deficient mice (at least 2×10^{6} cells/recipient). Injection was done by Edina Schweighoffer and ratios were confirmed by flow cytometry. Recipient Rag1-deficient animals were irradiated with 5 Gy using a ^{137}Cs-source, prior to injection. Chimeric animals received Baytril in their drinking water (0.02 %, Bayer Healthcare) for at least 4 weeks and were analysed by flow cytometry from 6 weeks on after transplantation.

2.3 Immunisations and anaesthesia

Mice were injected i.p. with PBS containing 10^{9} SRBC (Innovative Research), or 50 µg 4-hydroxy-3-nitrophenylacetyl $(NP)_{(0.6)}$-LPS in PBS, or 50 µg $NP_{(40)}$-AECM-Ficoll in PBS, or 50 µg $NP_{(21)}$-CGG (all BioSearch Technologies) in Alum (Thermo Scientific) with PBS for primary immunisations, or 50 µg $NP_{(21)}$-CGG in PBS for rechallenge and were bled or sacrificed at various time points for analysis or cell sorting. For cholera toxin immunisation, food was withdrawn from mice 3 h before being orally gavaged first with 1.5 % $NaHCO_3$ and then with 125 *µ*g/kg cholera toxin from *Vibrio cholerae* Inaba 569B (List Biological Laboratories) in PBS.

Blood was withdrawn at the indicated time points. Mice were sacrificed 14 d after immunisation to obtain faecal samples from the small intestine.

The acute house dust mite (HDM) model was performed together with Mark Wilson and mice were immunised i.p. with 10 μg crude HDM (Greer) in PBS with 25 % Alum (Thermo Scientific) on day 0 and day 14. For intratracheal challenges on day 28 and 31 mice were anaesthetised with a mix of 1 mg/mL ketamine with 0.1 mg/mL mededomidine in water and injected 0.1 mL / 10 g body weight i.p. (10 mg/kg ketamine and 1 mg/kg mededomidine). For intratracheal challenge each mouse received 100 μg HDM in 20 μL PBS by intratracheal gavage under anaesthesia. Anaesthesia was reversed using i.p. injection of atipamezole at 500 μg/mL and 0.1 mL / 10 g (5 mg/kg). For terminal anaesthesia mice received pentobarbitone 25 mg/mL at 0.1 mL / 10 g body weight (250 mg/kg). Mice were terminally bled under terminal anaesthesia.

2.4 Preparation of single cell suspensions

Single cell suspensions of bone marrow were prepared by flushing the bone marrow out of the bone using a syringe. Single cell suspension of spleen, Peyer's patches, peripheral lymph nodes and mesenteric lymph nodes were prepared by pushing the organs through a 70 μm strainer and rinsed using medium. Blood was collected using heparinised capillaries and peritoneal washes were obtained by flushing the peritoneum with 5 mL of PBS CMF containing 5 mM EDTA. Cells were generally handled on ice and centrifuged at 326 g (1300 rpm) for 5 min at 4 °C and kept in

air-buffered Iscove's modified Dulbecco's medium (AB IMDM) with 2 % foetal calf serum (FCS) (Lonza). Lungs were pushed through a 70 μm strainer then live cells were purified using 40 % isotonic percoll (GE Healtcare) in AB IMDM, layering the cells on top and then spinning for 20 min at 873 g (2000 rpm) with low deceleration. The pellet was used for further processing. Where required, cell suspensions were treated with ACK lysis buffer for 2 min at room temperature to remove red blood cells.

2.5 Splenic B cell isolation

Single cell suspensions were prepared as in section 2.4 and splenic B cells were isolated by magnetic negative depletion using biotinylated antibodies against CD43, CD11c, CD11b, Gr-1, and 1.2 to 1.4 × 10^8 streptavidin-Dynabeads (Life Technologies) in MACS-buffer unless stated otherwise. Purities of the isolations were verified by flow cytometry and were generally around 95 %.

2.6 Flow cytometry, cell sorting and cell enrichment

Single cell suspensions were stained for 20 min in ice cold PBS containing LIVE/DEAD fixable near-IR or aqua dead cell stain (Life Technologies) and the appropriate, pre-titred antibodies. Cell numbers in the bone marrow are quoted per leg (1 femur and 1 tibia). Regulatory B cells were purified using the Miltenyi Biotec Regulatory B cell isolation kit with 24 h *in vitro* LPS stimulation and phorbol-12-myristate-13-acetate (PMA) and

ionomycin for the last 5 h of stimulation followed by flow cytometric sorting for $B220^+$ $CD19^+$ $IL\text{-}10^+$ cells (Supplementary Figure 1). To isolate plasma cells and plasmablasts, organ suspensions from mice immunised 5 days earlier with SRBC were enriched for $CD138^+$ cells by staining with anti-CD138-PE and then using anti-PE beads according to the manufacturer's instructions (Miltenyi Biotec) followed by sorting for $B220^-$ $CD138^+$ cells for plasma cells and $B220^+$ $CD138^+$ cells for plasmablasts. Regulatory B cells, plasmablasts and plasma cells were enriched in AutoMACS buffer using the AutoMACS Pro (Miltenyi Biotec). To isolate germinal centre B cells, splenocytes from mice immunised 10 days earlier with SRBC were depleted using anti-CD43-biotin and anti-IgD-biotin followed by streptavidin-Dynabeads and then sorted for $B220^+$ PNA^+ $GL7^+$ Fas^+ $CD38^{low\text{-}int}$. Cell identities of antigen-experienced cell types was verified by qRT-PCR for upregulation of lineage transcription factors or cytokines (Supplementary Figure 2). Data were collected on a BD FACSCanto II or sorted on BD FACSAria II, BD Influx or Beckman Coulter MoFlo XDP cell sorters. Data were analysed using FlowJo 9.6 (TreeStar).

Table 1: Antibodies and staining reagents for flow cytometry and cell enrichment

Target	Conjugation	Supplier	Cat. No.	Clone	Isotype	Dilution	[µg/mL]
CD1d	AlexaFluor 488	eBioscience	53-0011-82	1B1	Rat IgG2b, κ	1:100	5.0
CD2	PE	BD Pharmingen	553112	RM2-5	Rat IgG2b, λ	1:800	0.25
CD3	AF488	BioLegend	100210	17A2	Rat IgG2b, κ	1:200	2.5
CD4	PerCPCy5.5	BioLegend	100434	GK1.5	Rat IgG2b, κ	1:200	1.0
CD4	PerCP	BioLegend	100538	RM4-5	Rat IgG2a, κ	1:200	1.0
CD4	eF450	eBioscience	48-0042-82	RM4-5	Rat IgG2a, κ	1:400	0.5

CD5	PE	eBioscience	12-0051-83	53-7.3	Rat IgG2a, κ	1:100	2.0
CD8α	BV570	BioLegend	100739	53-6.7	Rat IgG2a, κ	1:100	2.0
CD8α	PE	eBioscience	12-0081-83	53-6.7	Rat IgG2a, κ	1:200	1.0
CD8α	PerCP	BioLegend	100732	53-6.7	Rat IgG2a, κ	1:200	1.0
CD8α	PECy7	eBioscience	25-0081-82	53-6.7	Rat IgG2a, κ	1:400	0.5
CD8α	APC	eBioscience	17-0081-82	53-6.7	Rat IgG2a, κ	1:200	1.0
CD11b	PECy7	BioLegend	101216	M1/70	Rat IgG2b, κ	1:200	1.0
CD11b	PE	eBioscience	12-0112-82	M1/70	Rat IgG2b, κ	1:200	1.0
CD11b	Biotin	eBioscience	13-0112-85	M1/70	Rat IgG2b, κ	1:200	2.5
CD11c	Pacific Blue	BioLegend	117322	N418	Ar Ham IgG	1:200	2.5
CD11c	PE	eBioscience	12-0114-83	N418	Ar Ham IgG1, λ2	1:200	2.5
CD11c	Biotin	eBioscience	13-0114-85	N418	Ar Ham IgG1, λ2	1:200	2.5
CD19	PerCPCy5.5	BD Pharmingen	551001	1D3	Rat IgG2a, κ	1:200	1.0
CD19	APC	Life Technologies	RM7705	6D5	Rat IgG2a	1:100	2.0
CD19	eF450	eBioscience	48-0193-82	eBio1D3	Rat IgG2a, κ	1:200	1.0
CD23	PE	eBioscience	12-0232-83	B3B4	Rat IgG2a, κ	1:400	0.5
CD23	APC	Life Technologies	MCD2305	B3B4	Rat IgG2a, κ	1:200	1.0
CD38	FITC	eBioscience	11-0381-85	90	Rat IgG2a, κ	1:200	2.5
CD38	PECy5	eBioscience	15-0381-82	90	Rat IgG2a, κ	1:200	2.5
CD43	PE	BD Pharmingen	553271	S7	Rat IgG2a, κ	1:200	1.0
CD43	Biotin	eBioscience	13-0431-85	eBioR2/60	Rat IgM	1:200	2.5
CD45	APCCy7	BioLegend	103116	30-F11	Rat IgG2b, κ	1:200	1.0
CD45.1	FITC	eBioscience	11-0453-85	A20	Rat IgG2a, κ	1:200	2.5
CD45.2	PECy7	BioLegend	109830	104	Mouse (SJL) IgG2a, κ	1:200	1.0
CD69	FITC	BD Pharmingen	553236	H1.3F2	Ar Ham IgG1, λ3	1:200	2.5
CD86	PE	BD Pharmingen	553692	GL1	Rat IgG2a, κ	1:400	0.5
CD93 (AA4.1)	APC	eBioscience	17-5892-83	AA4.1	Rat IgG2b, κ	1:100	2.0
CD138	PE	BD Pharmingen	553714	281-2	Rat IgG2a, κ	1:200	1.0
B220	PE	eBioscience	12-0452-85	RA3-6B2	Rat IgG2a, κ	1:200	1.0
B220	PerCP	BD Pharmingen	553093	RA3-6B2	Rat IgG2a, κ	1:200	1.0
B220	PerCPCy5.5	BioLegend	103236	RA3-6B2	Rat IgG2a, κ	1:200	1.0
B220	PETR	Life Technologies	RM2617	RA3-6B2	Rat IgG2a, κ	1:200	1.0
B220	eF450	eBioscience	48-0452-82	RA3-6B2	Rat IgG2a, κ	1:200	1.0
B220	BV570	BioLegend	103237	RA3-6B2	Rat IgG2a, κ	1:200	0.5
CXCR5	Biotin	BD Pharmingen	551960	2G8	Rat IgG2a, κ	1:100	5.0
F4/80	APC	eBioscience	17-4801-82	BM8	Rat IgG2a, κ	1:100	2.0
Fas (CD95)	PE	BD Pharmingen	554258	Jo2	Ar Ham IgG2a, λ2	1:200	1.0
GL7 (Ly77)	AlexaFluor 647	BD Pharmingen	553666	GL7	Rat IgM, κ	1:100	2.0
Gr-1	Biotin	eBioscience	13-5931-85	RB6-8C5	Rat IgG2b, κ	1:200	2.5
IgD	PE	eBioscience	12-5993-83	11-26c	Rat IgG2a, κ	1:200	1.0
IgD	eF450	eBioscience	48-5993-82	11-26c	Rat IgG2a, κ	1:200	1.0
IgD	Biotin	eBioscience	13-5993-85	11-26c	Rat IgG2a, κ	1:200	2.5
IgM Fab	FITC	Jackson ImmunoResearch	115-097-020	polyclonal	goat Fab	1:300	0.5
IgM	PerCP	Santa Cruz	Sc-45094	Polyclonal	Goat	1:200	2.0
IgM	PECy5	eBioscience	21-5790-82	II/41	Rat IgG2a, κ	1:200	1.0
IgM	PECy7	eBioscience	25-5790-82	II/41	Rat IgG2a	1:200	1.0
IgM $F(ab)_2$	Biotin	Jackson ImmunoResearch	115-066-075	polyclonal	Goat $F(ab)_2$	1:220	5.0
IgG1	APC	BD Pharmingen	550874	X56	Rat IgG1, κ	1:200	1.0
Ly6C	PerCPCy5.5	BioLegend	128012	HK1.4	Rat IgG2c, κ	1:200	1.0
Ly6G	Pacific Blue	BioLegend	127612	1A8	Rat IgG2a, κ	1:200	2.5
MHC class II	eF450	eBioscience	48-5321-82	M5/114.15.2	Rat IgG2b, κ	1:400	0.5

NK1.1	APC	eBioscience	17-5941-82	PK136	Rat IgG2a, κ	1:200	1.0
PD-1 (CD279)	BV421	BioLegend	135217	29F.1A12	Rat IgG2a, κ	1:200	1.0
PNA	FITC	Vector Laboratories	FL-1071	-	-	1:1000	5.0
Siglec-F	PE	BD Pharmingen	552126	E50-2440	Rat IgG2a, κ	1:200	1.0
Strept-avidin	PE	BD Pharmingen	554061	-	-	1:200	2.5
TCRβ	FITC	eBioscience	11-5961-85	H57-597	Ar Ham IgG1, λ1	1:200	2.5
TCRβ	PE	eBioscience	12-5961-83	H57-597	Ar Ham IgG1, λ1	1:200	1.0
TCRβ	APC	eBioscience	17-5961-83	H57-597	Ar Ham IgG1, λ1	1:100	2.0
TCRγδ	PE	eBioscience	12-5711-82	eBioGL3	Ar Ham IgG2, κ	1:200	1.0

2.7 FACS-Gal

Spleen, peritoneal exudate and bone marrow cells were obtained as above and FACS-Gal assay was performed as described previously (Guo and Wu, 2008) using the substrate fluorescein di-(β-D-galactopyranoside) (FDG) (Sigma-Aldrich) and the inhibitor 2-Phenylethyl β-D-thiogalactoside (PETG) (Sigma-Aldrich). Briefly, ACK-lysed single cell suspensions were resuspended in 100 μL at 2×10^7 cells/mL in Hank's balanced salt solution (HBSS), pre-warmed for 10 min at 37 °C in a water bath and then mixed with 100 μL of equally pre-warmed 20 mM FDG in dH_2O. FDG loading (at 10 mM FDG final concentration) was stopped after 1 min by addition of 2 mL ice-cold HBSS. For catalysis of FDG into fluorescein and galactose by β-galactosidase, cells were left on ice for 45 min unless indicated otherwise. The reaction was then stopped by adding 44 μL of 50 mM PETG (1 mM final concentration). Cells were then stained for flow cytometry as described above.

2.8 BCR internalisation assay

Single cell suspensions of splenocytes were stained on ice with anti-IgM $F(ab)_2$-biotin at 10 mg/mL then aliquots were kept in a 37 °C water bath for the indicated times and BCR internalisation was quenched with ice-cold PBS. Remaining surface BCRs were stained with streptavidin-PE and revealed by flow cytometry.

2.9 Antigen presentation assay

Splenic B cells from *Themis2*$^{KO/KO}$ or wild type littermate control mice were isolated using magnetic negative depletion with anti-CD43-PE, anti-CD11b-PE, anti-CD11c-PE and anti-TCRβ-PE antibodies and anti-PE beads (Miltenyi Biotec) according to the manufacturer's instructions. Splenic and lymph node $CD4^+$ T cells from OT-II Rag1-deficient mice were isolated similarly but using anti-CD11b-PE, anti-CD11c-PE, anti-CD8-PE, anti-B220-PE. To activate the B cells and deliver the antigen to the BCR, B cells were stained with 2.5 or 0.25 or 0.025 or nil mg/mL anti-IgM $F(ab)_2$-biotin and then with OVA antigen delivery reagent (Miltenyi Biotec) according to the manufacturer's instructions. T cells were labelled with 5 mM CellTrace Violet (CTV) (Life Technologies) in Dulbecco's PBS (Gibco) for 20 min at 37 °C. Purities of cell isolations and loading of CTV and antigen were checked by flow cytometry. 2×10^5 B cells were co-cultured with 10^5 OT-II $CD4^+$ T cells in 96-well plates for 72 h unless indicated otherwise. T cell proliferation was assessed by CTV dilution via flow cytometry and IL-2 was measured in the supernatants by ELISA.

2.10 RNA preparation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from single cell suspensions was extracted with the RNEasy Mini or Micro Plus kits (Qiagen) according to the manufacturer's instructions. Gut and spinal cord tissue were pre-processed using the total RNA setting on a GentleMACS Dissociator (Miltenyi Biotec). Lung RNA was obtained by mashing the tissue using metal beads in Qiazol (Qiagen) using a Precellys 24 tissue homogenizer with Cryolys cooling (Bertin). Lung RNA was extracted from the homogenate first by chloroform extraction, followed by addition of ethanol to the chloroform phase (chloroform 2:7 ethanol), RNA binding of this mixture to an RNEasy Mini spin column (Qiagen) and subsequent purification according to the manufacturer's instructions. cDNA was synthesised using the Superscript III First Strand Synthesis SuperMix for qRT-PCR kit (Life Technologies) according to the manufacturer's instructions. Samples were analysed on an ABI Fast 7900HT under standard conditions using the TaqMan Gene Expression Mastermix and Gene Expression Assays *Themis2* exon 2-3 Mm01180768_m1, *Themis2* exon 3-4 Mm01180769_m1, *Themis2* exon 4-5 Mm01180770_m1, *Themis1* Mm00724485_m1, *Themis3* Mm01239731_m1, *Hprt1* Mm03024075_m1, *Garem* Mm01158214_m1, *Garem1* Mm01210799_m1 or the custom designed assays *Themis2* exon 3-5 AJ1RUPJ, *Themis2* exon 5-6 AJGJPQS (all Life Technologies) according to the manufacturer's instructions. qRT-PCRs from lung tissue were performed using the QuantiTect SYBR Green PCR kit (Qiagen) with

primers in the table below at 1 μM concentration on an ABI Fast 7900HT under the following conditions 95 °C 15 min, 40 cycles of 94 °C 15 s, 55 °C 30 s, 72 °C 30 s followed by 1 cycle of 95 °C 15 s, 60 °C 15 s, 95 °C 15 s for a 95 °C to 60 °C dissociation curve. All data were normalised to *Hprt1* and analysed using the comparative C_T method.

Table 2: List of qRT-PCR primers

Primer	Sequence	T_m [°C]
Arginase 1 For	GGAAAGCCAATGAAGAGCTG	57.3
Arginase 1 Rev	GCTTCCAACTGCCAGACTGT	59.4
Eotaxin-1 (CCL11) For	AGAGCTCCACAGCGCTTCT	58.8
Eotaxin-1 (CCL11) Rev	GCAGGAAGTTGGGATGGAG	58.8
Fizz1 For	CCCTCCACTGTAACGAAGACTC	62.1
Fizz1 Rev	CACACCCAGTAGCAGTCATCC	61.8
Gob-5(ClCA3) For	CATCGCCATAGACCACGACG	61.4
Gob-5(ClCA3) rev	TTCCAGCTCTCGGGAATCAAA	57.9
HPRT1 For	GCCCTTGACTATAATGAGTACTTCAGG	63.4
HPRT1 Rev	TTCAACTTGCGCTCATCTTAGG	58.4
IFN-gamma For	ACAGCCAGATTATCTCTTTCTACCTCAG	57.5
IFN-gamma Rev	CCTTTTTCGCCTTGCTGTTG	57.3
IL-13 For	CCTCTGACCCTTAAGGAGCTTAT	60.6
IL-13 Rev	CGTTGCACAGGGGAGTCTT	58.8
IL-17A p2 F	ACCCTGGACTCTCCACCGCAA	63.1
IL-17A p2 R	GGCTGCCTGGCGGACAATCG	64.0
IL-4 For	ACGAGGTCACAGGAGAAGGGA	61.8
IL-4 Rev	AGCCCTACAGACGAGCTCACTC	64.0
IL-5 For	TGACAAGCAATGAGACGATGAGG	60.6
IL-5 Rev	ACCCCCACGGACAGTTTGATTC	62.1
MUC5AC For	CAGGACTCTCTGAAATCGTACCA	60.6
MUC5AC Rev	AAGGCTCGTACCACAGGGA	58.8
Ym1 For	CATGAGCAAGACTTGCGTGAC	59.8
Ym1 Rev	GGTCCAAACTTCCATCCTCCA	59.8

2.11 RNA sequencing and data analysis

Follicular B cells were sorted by flow cytometry from the spleens of *Themis2*$^{KO/KO}$ or wild type littermate control mice. Unstimulated samples were lysed directly, stimulated samples were cultured as described under cell culture at 3×10^6 cells/mL for 6 h with either 10 mg/mL anti-IgM, or 10 mg/mL LPS or 1 mg/mL CD40L with 100 ng/mL IL-4. RNA was isolated using Trizol reagent (Life Technologies) according to the manufacturer's instructions and cleaned up using RNEasy mini kit (Qiagen). Single end, unstranded, poly-A^+-enriched libraries were made using the TruSeq RNA sample preparation kit (Illumina). Samples were first checked for RNA quality, then library quality using the Agilent Bioanalyzer and subsequently sequenced with an Illumina HiSeq 2000, collecting 13.2 – 76.1 million reads of 75 bases per sample (Supplementary Figure 3A). Quality control and sequencing was performed by the NIMR high throughput facility. Avadis NGS (V1.4.7) was used for VDJ analysis and reads were mapped to NCBI37/mm10 genome and transcriptome including an update containing additional VDJ annotation derived from GenBank entries provided by Avadis NGS. Aligned reads were mapped to the RefSeq transcripts database and were normalised by DESeq (Anders and Huber, 2010). Computational analysis of differential gene expression was done by Nikolay Nikolov and reads were aligned to mm10 (Ensembl version 72) using Tophat (version 2.0.9). Raw counts were determined using the union method in htseq-count (version 0.5.4p3) and mappings were filtered for a phred score of > 10. edgeR (Version 3.2.4) was used for filtration of lowly

expressed features, normalisation and statistical analysis. Statistical significance of differences in gene expression was determined using the exact binomial test, reporting differences with a false discovery rate (FDR) < 0.05 (Robinson and Smyth, 2008). Gene expression is presented as read density defined as normalised counts per kilobase of exon. Pathway analysis was performed using Ingenuity IPA. All RNAseq data is deposited under the accession number E-MTAB-2499 in ArrayExpress (https://www.ebi.ac.uk/arrayexpress).

2.12 Immunoblot

Splenocyte suspensions were treated with ACK lysis buffer for 2 min at room temperature, incubated with biotinylated anti-CD43 and negative-depleted using magnetic streptavidin-Dynabeads. Purities of the B cell isolations were verified by flow cytometry and were generally around 95 %. Cell lysates were prepared using RIPA buffer. Cell debris were removed by centrifugation and SDS sample buffer was added to the cleared lysates followed by denaturation at 95 °C. for 5 min. Proteins were separated at 100 V on 7 % SDS-PAGE mini gels in SDS-PAGE running buffer and then wet-blotted using CAPS buffer onto Immobilon-FL PVDF membrane (Millipore) at 350 mA for 1 h by standard techniques. Membranes were blocked for 1 h in Odyssey Blocking Buffer (Li-Cor #927-40000), and then probed with the following antibodies over night at 4 °C in Odyssey Blocking Buffer: anti-THEMIS2, ICB1 (P-14), goat polyclonal, Santa Cruz #sc-160439; anti- THEMIS2, ICB1 (G-15), goat polyclonal, Santa Cruz

#sc-160437; anti- THEMIS2, ICB1 (D-14), goat polyclonal, Santa Cruz #sc-160436; anti- THEMIS2, ICB1 (C-term), rabbit polyclonal, Abgent #AP9910b. Then, membranes were incubated with the appropriate secondary antibodies: Alexa-Fluor680 goat anti-rabbit IgG (H+L), Invitrogen #A21109 or IRDye700DX-conjugated donkey anti-goat IgG (H+L), Rockland #605-730-125 and signals were detected using the Odyssey Infrared Imager (Li-Cor Biotechnology). To check for loading membranes were then incubated over night at 4 °C in Odyssey Blocking Buffer with anti-α-TUBULIN mouse monoclonal (clone TAT-1), prepared in-house and subsequently with the secondary antibody goat anti-mouse IRDye800CW, Licor Biosciences #926-32210. Membranes were washed three times with PBS containing 0.05 % Tween20 for at least 5 min on a shaker at room temperature in between antibody incubation steps and measurements. Signals were again detected with an Odyssey Infrared Imager (Li-Cor Biotechnology) and analysed with the manufacturer's software.

2.13 ELISAs

All plates were developed using 3,3′,5,5′-tetramethylbenzidine (TMB) (eBioscience) and stopped with 1 M H_2SO_4. Signals were quantified using absorption at 450 nm measurement wavelength and 570 nm reference wavelength on a Tecan Sapphire^2. Data was analysed in Microsoft Excel and Graphpad Prism 6. Only values of serial dilutions in the linear portion of the response curve were used to calculate analyte levels.

2.13.1 Sample taking

Blood samples were taken and left to clot for at least 10 min, then centrifuged twice at 17000 g to pellet cells. Serum supernatants were transferred into a new tube and then stored at -20 °C for short-term or -70 °C for long-term storage. Cell culture supernatants were obtained by prior centrifugation at 300 g to spin down cells. Supernatants were frozen at -70 °C until analysis by ELISA. Faecal samples from the small intestine were weighed, diluted with PBS 1 mL / 0.1 g faeces centrifuged for 5 min at 400 g at 4 °C to remove large debris and then for 10 min at 9000 g at 4 °C to pellet bacteria. Supernatants were frozen at -70 °C until analysis.

2.13.2 Total, NP-specific and cholera-toxin-specific immunoglobulin ELISAs

Total serum immunoglobulin levels were quantified by ELISA with the SBA Clonotyping System-B6/C57J-HRP (SouthernBiotech) on 96-well Maxisorp Immunoplates (Nunc) according to the manufacturer's instructions. Briefly, plates were coated over night with 10 μg/mL AffiniPure Goat Anti-Mouse IgG + IgM (H + L) (Jackson Labs) and blocked with 3 % bovine serum albumin (Sigma-Alrdich) in PBS for 2 h at room temperature. Afterwards serial dilutions of sera and standards in PBS were incubated for 2 h at room temperature and HRP-conjugated antibodies were diluted 1:4000 in PBS with 0.05 % Tween20 (PBST) and incubated for 1 h at room temperature. Plates were washed three times in PBST by immersion in between steps. Both NP-specific ELISAs and cholera-toxin specific

ELISAs were performed analogously but plates were coated with 5 μg/mL NP_{18}-BSA (BioSearch Technologies) or 1 μg/mL cholera toxin from *Vibrio cholerae* Inaba 569B (List Biological Laboratories) respectively.

2.13.3 Total IgE ELISAs

96-well Maxisorp Immunoplates (Nunc) or 96-well ELISA half area Microplate, flat bottomed, high binding (Greiner Bio-One) were coated with 2 μg/mL purified RAT Anti-Mouse IgE antibody (BD Bioscience, Clone R35-72) in 60 mM carbonate buffer pH 9.6 over night at 4 °C. Plates were washed with PBST and then blocked with 5 % skimmed milk in PBS for 2 h at 37°C an then washed again. Sera and standards were serially diluted in PBS and applied to the plate for 1 h at 37 °C. Plates were washed with PBS and 1 μg/mL biotinylated rat anti-mouse IgE (BD Bioscience, Clone R35-118) diluted in 5 % skimmed milk in PBST was added for 1 h at room temperature. After washing, 0.5 μg/mL streptavidin-conjugated HRP (KPL) diluted in 5 % milk in PBST was added and incubated for 30 min at room temperature. Plates were washed seven times with PBST and then twice with purified water before addition of the substrate TMB. Plates were incubated in the dark until developed sufficiently.

2.13.4 Anti-X31 haemagglutinin IgG-ELISA

For determination of anti-X31 haemagglutinin (HA)-IgG, 96-well flat bottomed plates were coated with 2.5 mg/ml of purified X31 HA (a kind gift from Dr. John Skehel, NIMR) in PBS and incubated overnight at 4 °C. Plates were blocked with 2 % BSA in PBS for 2 h at room temperature then serial dilutions of heat inactivated sera samples were incubated for 3 h at 37 °C. Then X31 HA-specific IgG was detected with HRP-conjugated goat anti-mouse IgG antibody (BioRad) diluted 1:3000. Plates were washed with PBST in between steps.

2.13.5 IL-2 ELISA

The DuoSet mouse IL-2 ELISA (R&D systems) was used according to the manufacturer's instructions with the following modifications: Plates used were 96-well ELISA half area Microplate, flat bottomed, high binding (Greiner Bio-One), Streptavidin-HRP was from BD Biosciences and used 1:1000 and plates were washed 10 times before the addition of the substrate.

2.14 Cell culture

Single cell suspensions of purified B cells were prepared as described in section 2.5, cultured in DMEM-plus medium (DMEM with 100 µM non-essential amino acids, 20 mM HEPES buffer (all Gibco) 10 % FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 2 mM L-Glutamine, and

100 μM 2-mercaptoethanol (all Sigma-Aldrich)). Where not indicated otherwise, cells were stimulated with the reagents at the given concentration in the table below.

Table 3: List of stimulatory reagents

Reagent	Provider	Cat-No	Concentration
Anti-IgM $F(ab)_2$	Jackson ImmunoResearch	115-006-075	10 μg/mL
LPS	Alexis	Alx-581-008-L002	10 μg/mL
CD40L	R&D Systems	1163-CL	1 μg/mL
IL-4	PeproTech	214-14	100 ng/mL
BAFF	PeproTech	310-13	100 ng/mL
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich	P1585-10MG	50 ng/mL
Ionomycin	Sigma-Aldrich	I06354	500 ng/mL

2.14.1 Cell surface activation marker measurement

Cells were cultured as described in section 2.14 in flat bottom 96-well plates at 4×10^5 cells/well and stimulated with the indicated stimuli for 24 h. Then cells were stained and analysed by flow cytometry for surface levels of CD23, CD69, CD86 and MHC class II as described in section 2.6

2.14.2 Proliferation and survival assays

B cells were isolated and cultured as described in section 2.14 but labelled with 5 μM CFSE (Life Technologies) in Dulbecco's PBS (Gibco) for 10 min at 37 °C before culturing 10^6 cells/well in flat bottom 48-well plates for

72 h. Proliferation was assessed by CFSE dilution and number of live cells was measured by a LIVE/DEAD fixable near-IR dead cell stain and counted using PerCP-beads (BD Calibrite) on a BD FACS Canto II flow cytometer. Data were analysed using the cell proliferation platform in FlowJo 9.6.

2.14.3 Luminex cytokine assay

B cells were isolated and cultured as described in section 2.14 in flat bottom 96-well plates at 4×10^5/well for 72 h. Then supernatants were harvested and frozen at -70 °C until measurement of 16 cytokines (CCL2, GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p70, IL-13, IL-17A, KC, MIP-2, TNFα, VEGF) using the Luminex-based 16-plex Fluorokine MAP assay (R&D Systems) according to the manufacturer's instructions on a Luminex 100 machine (Luminex).

2.15 Influenza infection

Mice were infected intranasaly with 30 μL PBS containing X31 influenza $TCID_{50} = 8 \times 10^3$ or 8×10^4 by Sophia Davidson and monitored daily for weight loss and clinical symptoms for 15 days. Mice were bled on day 10 for measurement of anti-X31 HA IgG antibody levels. Mice that reached 75 % of their starting weight were euthanised complying with UK home office regulations.

2.16 Cytospins

Cytospins were performed by Mark Wilson on cells retrieved from bronchoalveolar lavages performed using 1.5 mL ice-cold PBS. Cytospins were prepared using a Cytospin 4 (Thermo Scientific) at 1200 rpm for 5 min. Cells were fixed in methanol and then stained with modified Accustain Wright-Giemsa stain (Sigma-Aldrich) to identify cells morphologically. Cells were counted manually using a Leica DM1000 LED microscope.

2.17 Histology

For histopathological analysis the left lung lobe was removed and fixed in 4 % paraformaldehyde in PBS for 1 day then transferred to 70 % ethanol for 1 day. The NIMR Histology facility embedded the samples in paraffin, cut into 5 μm sections and stained with hematoxylin and eosin (H&E), or alcian blue-periodic acid schiff, as indicated. Samples were and analysed on an Olympus VS120 slide scanner by Mark Wilson.

2.18 Isoform PCRs and genotyping

For genotyping, genomic DNA was extracted from mouse tail or ear clippings by lysis in a proteinase K (Roche)-containing tail lysis buffer followed by isopropanol precipitation of the gDNA. Genotyping PCRs for *Themis2* alleles were developed using Primer3 (Rozen and Skaletsky, 2000) and Primer-BLAST (Sayers et al., 2011) and were optimised for

annealing temperature by gradient PCR and titrated for optimal $MgCl_2$ concentration. All PCRs were performed using Thermoprimer Plus DNA Polymerase (Thermo Scientific) in Reaction Buffer IV (Thermo Scientifc) on a DNA Engine Dyad Peltier Thermal Cycler (Biorad). Reactions were separated in 1 – 3 % agarose in TAE buffer gels.

PCRs for the detection of isoforms of *Themis2* were performed on sorted, follicular B cell cDNA. Four different primer pairs annealing in exon 1 and exon 6 of the full-length *Themis2* transcript were used to identify shorter transcripts. Detailed protocols and primer sequences for all reactions can be found in the appendix. An example of each genotyping reaction can be found in Supplementary Figure 4.

2.19 Statistical analysis

Statistical significance of differences between groups was assessed using the Mann-Whitney test or chi-square test where applicable in GraphPad Prism 6 or the Fisher's exact test in Ingenuity IPA. Differences with $p < 0.05$ were considered significant.

2.20 Solutions, buffers, media and gels

Table 4: Composition of solutions, buffers, media and gels

Solution	Composition
AB IMDM	2.1 g NaCl, 0.06 g Penicillin, 0.1 g Streptomycin, IMDM powder for 1 L, 1 L dH_2O, adjust to pH 7.2, sterilise by membrane filtration
ACK lysis buffer	155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, 1 L dH_2O
AutoMACS buffer	AB IMDM, 2 mM EDTA, 2 % FCS, sterile filtered
CAPS buffer	4.4 g/L CAPS (3-[cyclohexylamino]-1-propanesulphonic acid) in dH_2O, pH 11.0
DNA Loading buffer	20 % Ficoll 40, 0.1 M Na_2EDTA pH 8, 1 % SDS, 0.25 % Bromophenol blue
RIPA lysis buffer	150 mM NaCl, 1 % NP40, 0.25 % Sodiumdeoxychelate, 2 mM EDTA, 50 mM Tris pH 7.4, 1 mM DTT, 1 mM $NaVO_3$, 1 % Protease Inhibitor p8340 (Sigma-Aldrich)
FACS buffer	PBS, 0.1 % NaN_3, 0.5 % BSA
MACS buffer	PBS, 2 mM EDTA, 0.5 % BSA
PBS	8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 1 L dH_2O
PBST	PBS, 0.05 % Tween20
SDS Sample buffer	0.2 M Tris-HCl, 3 % SDS, 10 % glycerol, 3 % 2-Mercaptoethanol, 0.25 ‰ Bromophenol blue, pH 6.8
TAE	10 mM Tris-HCl, pH 7.4, 0.02 M Glacial Acetic Acid, 1 mM EDTA, pH 8.0
Lower buffer	1.5 M Tris-HCl, 0.4 % SDS, pH 8.8
Upper buffer	0.5 M Tris-HCl, 0.4 % SDS, pH 6.8

10× SDS-PAGE running buffer	3.03 M Tris-HCl, 1 % SDS, 14.42 % Glycine, pH 8.3
7% SDS-PAGE mini gel	Resolving gel: 1.75 mL Protogel 30 % Acrylamide 0.8 % Bis-Acrylamide (National Diagnostics), 1.88 mL Lower Buffer, 3.87 mL dH_2O, 5 μL Tetramethyl-ethylenediamine (TEMED) and 25 μL Ammonium Persulfate (APS) 10 %; let polymerise under water-saturated Butanol then add Stacking Gel: 0.4 mL Protogel (as above), 0.8 mL Upper Buffer, 1.9 mL dH_2O, 3.125 μL TEMED and 15.625 μL APS 10 %

3 Results

In order to determine the function of *Themis2* in B cells I first sought to establish a *Themis2*-deficient mouse strain which would allow analysis of B cell development and activation *in vivo* as well as *in vitro*. At the beginning of this study, neither the *Themis2*-deficient mouse strain described here, nor any other had been described in literature.

3.1 Generation of a *Themis2*-deficient mouse strain

Matthew Pierce from Imperial College London generated a mouse strain with the targeted allele *Themis2*$^{tm1a(KOMP)Wtsi}$, here further termed *Themis2*^{NeoFlox}, from embryonic stem cells of the Wellcome Trust Sanger Institute's KOMP repository. I received *Themis2*$^{WT/NeoFlox}$ mice on a C57BL/6 background, which contain a construct in the *Themis2* gene targeting exon 4 as depicted in Figure 5. Exon 4 is flanked by loxP sites for recombination with a Cre recombinase. 5' of exon 4 a promoterless reporter cassette has been inserted allowing for selection using a neomycin resistance gene and detection of endogenous *Themis2* promoter activity using a β-galactosidase reporter gene. Splicing into the reporter cassette is induced by an EN2 splice acceptor site thus transcribing both genes from the endogenous *Themis2* promoter. Both genes are expressed as non-fusion proteins due to self-cleaving T2A oligopeptide sequences. The whole cassette is flanked by FRT sites and

can be deleted using FLP recombinase. Although this allele is termed a "knockout-first" allele by KOMP, I decided to breed a complete null allele as data suggested that there was still residual THEMIS2 produced. Although strongly reduced on both protein and mRNA levels, I could still detect residual THEMIS2 protein by immunoblot and I could detect splicing from *Themis2* exon 3 to exon 4 and from exon 4 to exon 5 by qRT-PCR in *Themis2*$^{NeoFlox/NeoFlox}$ mice (data not shown). In two sequential steps I first crossed *Themis2*$^{+/NeoFlox}$ mice to C57BL/6J-Tg(Prm-cre)70Og mice which have Cre under control of the protamine 1 promoter. This leads to deletion of *Themis2* exon 4 in the male germline as the protamine 1 promoter drives Cre recombinase expression in male gametes (O'Gorman et al., 1997). The newly produced allele, in which *Themis2* exon 4 is deleted but the reporter cassette is still functional, is called *Themis2*$^{tm1b(KOMP)Wtsi}$ and is here further referred to as *Themis2*^{NeoKO}. Subsequently, *Themis2*$^{+/NeoKO}$ mice were bred with C57BL/6J-Tg(ACTFLPe)9205Dym mice which have a heat-stable form of the recombinase FLP, termed FLPe, under the control of the human *ACTB* promoter. This cross efficiently removed the reporter cassette, leaving behind only an FRT- and a loxP-site in between *Themis2* exon 3 and exon 5, creating the *Themis2*$^{tm1d(KOMP)Wtsi}$ allele, henceforth called *Themis2*^{KO}. *Themis2*$^{KO/KO}$ mice were used for all experimental work presented here with the exception of the data on *Themis2* promoter activity for which *Themis2*$^{+/NeoFlox}$ mice were used. FLPe and Cre genes were removed by backcrossing and experimental mice (*Themis2*$^{KO/KO}$) were bred from the intercross of *Themis2*$^{+/KO}$ parents to produce both

mutant and littermate control (WT, *Themis2*$^{WT/WT}$) animals. Initial data on B cell numbers during development suggested greater data dispersion if *Themis2*$^{KO/KO}$ mice were compared to in house C57BL/6J-Nimr controls (a genetically drifted colony of C57BL/6J that was established at NIMR about 40 years ago). The variability could have arisen from the fact that the embryonic stem cell line from which the *Themis2* alleles originated was on a C57BL/6N background and mice were not fully backcrossed to C57BL/6J-Nimr. To minimise biological variability, I decided to only use age- and sex-matched littermate controls for the determination of *Themis2* phenotypes.

The targeted exon in Themis2^{KO} mice, exon 4, is the biggest exon of *Themis2* as illustrated in Supplementary Figure 5. Deletion of *Themis2* exon 4 in *Themis2*$^{KO/KO}$ mice leads to loss of a substantial part of the protein. Exon 4 encodes the C-terminal part of the first CABIT domain, all of the second CABIT domain, the NLS and the proline-rich SH3-binding site. Furthermore, as exon 4 starts and ends in different phases, deletion of exon 4 leads to a frameshift from exon 3 into exon 5. Consequently, both exon 5 and exon 6 are then out of frame leading to loss of the complete C-terminus, including the SH2-binding site, in addition to all functional domains encoded by exon 4. Thus all that is left of the protein if exon 4 is deleted, is an N-terminal fragment of the first CABIT domain followed by a frameshifted C-terminal tail.

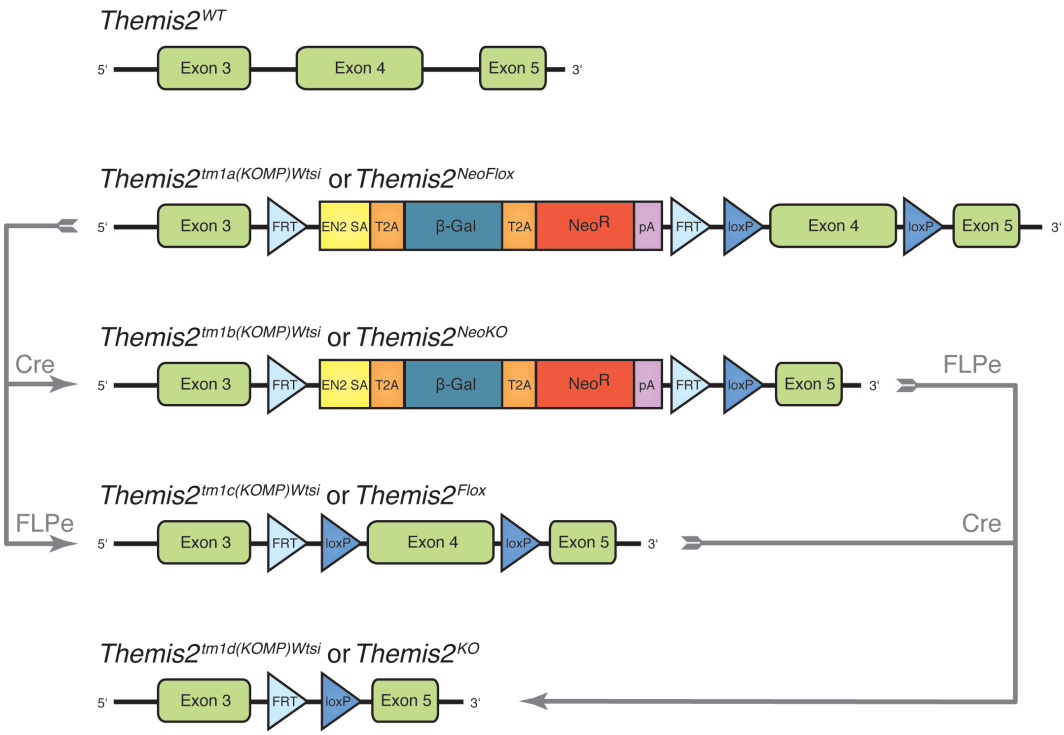

Figure 5: *Themis2* KOMP gene targeting strategy and derivation of *Themis2* alleles.

Naturally occurring *Themis2* wild type (WT) allele and the KOMP repository-derived, promoterless *Themis2*^{NeoFlox} allele are shown on top. The *Themis2*^{NeoFlox} allele contains a reporter cassette flanked by FRT-sites for recombination with a FLPe-deleter strain. The reporter cassette consists of an EN2 splice acceptor site (EN2 SA), followed by a T2A self-cleaving oligopeptide and the coding sequence (CDS) for the β-galactosidase reporter gene (β-Gal). Subsequently encoded are another T2A self-cleaving oligopeptide followed by a CDS for a neomycin-phosphotransferase (Neo^{R}) enabling selection with G418. Transcription is terminated using an SV40 polyadenylation site (pA). This setup allows discrete expression of THEMIS2 fragments, β-galactosidase and neomycin-phosphotransferase as non-fusion proteins though the self-cleaving oligopeptides. It also permits the assessment of the *Themis2* promoter-activity via measurement of β-galactosidase activity as the whole construct is promoterless and the β-galactosidase is under control of the endogenous *Themis2* promoter. Downstream of the reporter cassette exon

4 is flanked by two loxP sites for recombination using a Cre-deleter strain. Crossing of mice bearing a *Themis2*^{NeoFlox} allele to either a Cre- or FLPe-deleter strain results in creation of the *Themis2*^{NeoKO} or *Themis2*^{Flox} alleles respectively. Crossing of these strains with the opposite deleter strain leads to production of the *Themis2*^{KO} allele.

3.2 Expression of the Themis family in B cells

First, I measured the expression of Themis family members in B cells. On the one hand this served to expand and confirm publicly available expression data and determine possible B cell developmental or activation stages in which *Themis2* might be of particular importance. On the other hand this was necessary in order to determine if there was expression of other Themis family members that might functionally substitute for *Themis2* in its absence, as has been shown for other signalling molecules downstream of the BCR such as in the case of *Rac1/Rac2* or *Vav1/Vav2/Vav3* (Fujikawa et al., 2003; Walmsley et al., 2003).

3.2.1 Expression of the Themis family in B Cell subsets

I performed high throughput RNA sequencing on sorted mouse follicular B cells to directly compare the expression of CABIT-domain containing genes in the most abundant mature B cell subset. As depicted in Figure 6A, only *Themis2* is expressed at high levels whereas neither the other Themis-family members *Themis1* and *Themis3* nor the two other CABIT-domain containing proteins in the mouse genome, *Garem* and *Garem*l, are expressed to a significant degree. Next, I sorted developmental B cell subsets from the bone marrow (pro-B, pre-B, immature B,) and spleen (transitional type 1 (T1), T2, T3,) as well as mature, naïve B cell subsets from the bone marrow (recirculating, mature B cells), spleen (follicular and marginal zone B cells) and peritoneal cavity (B1 and B2 cells) and

analysed them by qRT-PCR for the expression of the Themis family as illustrated in Figure 6B. As positive controls I used RNA from thymus tissue, splenic and lymph node T cells for *Themis1* and RNA from the small intestine for *Themis3* respectively. Similarly, I sorted antigen-experienced B cells from the bone marrow (plasma cells) and from the spleen (plasmablasts, plasma cells, germinal centre B cells, IL-10-producing B regulatory cells) for analysis by qRT-PCR as shown in Figure 6C. Consistent with publicly available data and published studies (Lesourne et al., 2009) the results show that *Themis1* is only expressed in T cells and in the thymus but not in any B cell population. *Themis3* was not detectable in any sorted B or T cell population but readily detectable in the gut. *Themis2* is expressed in all analysed B cell populations. I also noted that germinal centre B cells have reduced expression of *Themis2* compared to follicular B cells ($p = 0.017$ in Mann Whitney), again in agreement with publicly available data from the Immgen consortium (www.immgen.org) (Heng and Painter, 2008). Moreover, I found reduced *Themis2* expression in regulatory B cells, which had been cultured and re-stimulated with LPS, PMA and ionomycin *in vitro* to induce IL-10 production ($p = 0.006$ in Mann Whitney). Overall, *Themis2* is expressed non-redundantly in the B cell lineage and its expression is reduced in antigen-experienced B cells.

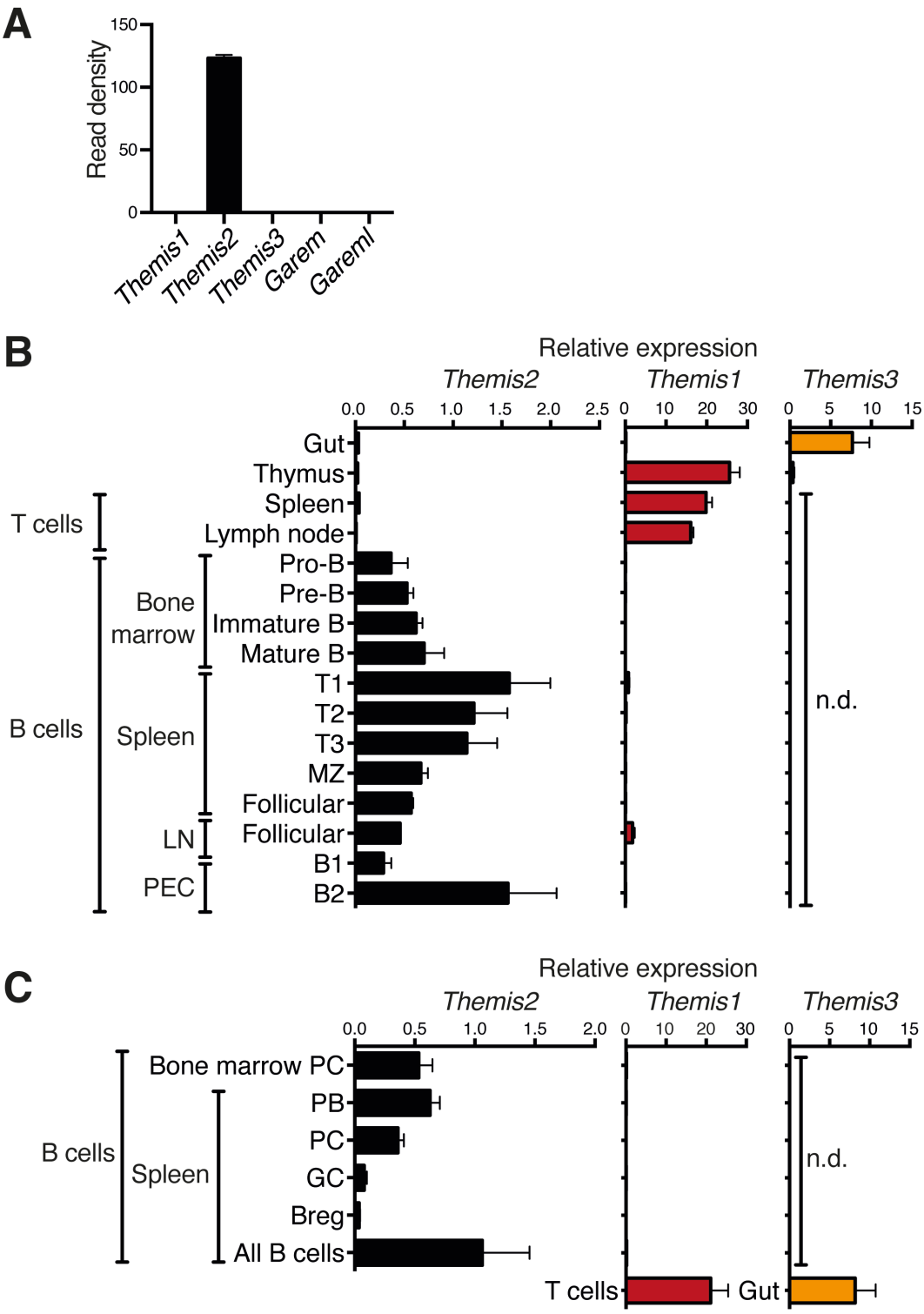

Figure 6: *Themis2* is expressed non-redundantly in the B cell lineage. **(A)** Mean ± SEM of mRNA expression of all mouse CABIT domain-containing proteins in follicular B cells sorted via flow cytometry and analysed by RNA sequencing. Data are displayed as read density. **(B)** Mean ± SEM of mRNA expression by quantitative real-time PCR (qRT-

PCR) analysis of Themis family members in whole organs or various T and B cell developmental populations sorted via flow cytometry. **(C)** Mean ± SEM of mRNA expression by qRT-PCR of Themis family members in antigen-experienced B cell populations. Germinal centre B cells, regulatory B cells, plasmablasts and plasma cells were isolated and sorted as described in section 2.6 of materials and methods. All other cell types were gated and sorted as depicted in Figure 12. All data are from ≥ 3 biological replicates per population and sorting purities generally were > 95 %. LN, lymph node; PEC, peritoneal exudate cells; MZ, marginal zone B cells; PC, plasma cells; PB, plasmablasts; GC, germinal centre B cells; Breg, regulatory B cell. n.d. not detected.

3.2.2 *Themis2* promoter activity in B cell subsets

To further corroborate *Themis2* expression in B cells I measured *Themis2* promoter activity in *Themis2*$^{+/NeoFlox}$ mice using the β-galactosidase reporter controlled by the endogenous *Themis2* promoter in a FACS-Gal assay. In this assay the non-fluorescent substrate FDG is converted into fluorescein by the activity of β-galactosidase. As transcription of the β-galactosidase gene is controlled by the endogenous *Themis2* promoter, the amount of β-galactosidase is assumed to be proportional to the amount of THEMIS2 produced. Kinetic measurements established that measurements were taken after an appropriate reaction time and before substrate depletion, allowing for discrimination of both higher and lower fluorescein production in the assay (Supplementary Figure 6A). *Themis2* promoter activity was detected in B cells and CD11c^{+} cells such as dendritic cells but not in T cell (Supplementary Figure 6B), consistent with publicly available expression data for *Themis2* (www.immgen.org, www.biogps.org). I found that developing and mature, naïve B cells with the *Themis2*^{NeoFlox} reporter construct showed strong promoter activity compared to wild type controls whereas reporter activity in B220^{-} cells in the spleen or in CD5^{+} T cells in the peritoneal cavity was low both in terms of mean fluorescence intensity of fluorescein and the percentage of fluorescein-positive cells (Figure 7). The FACS-Gal data further support previous findings that *Themis2* is highly expressed in B cells and dendritic cells but not T cells.

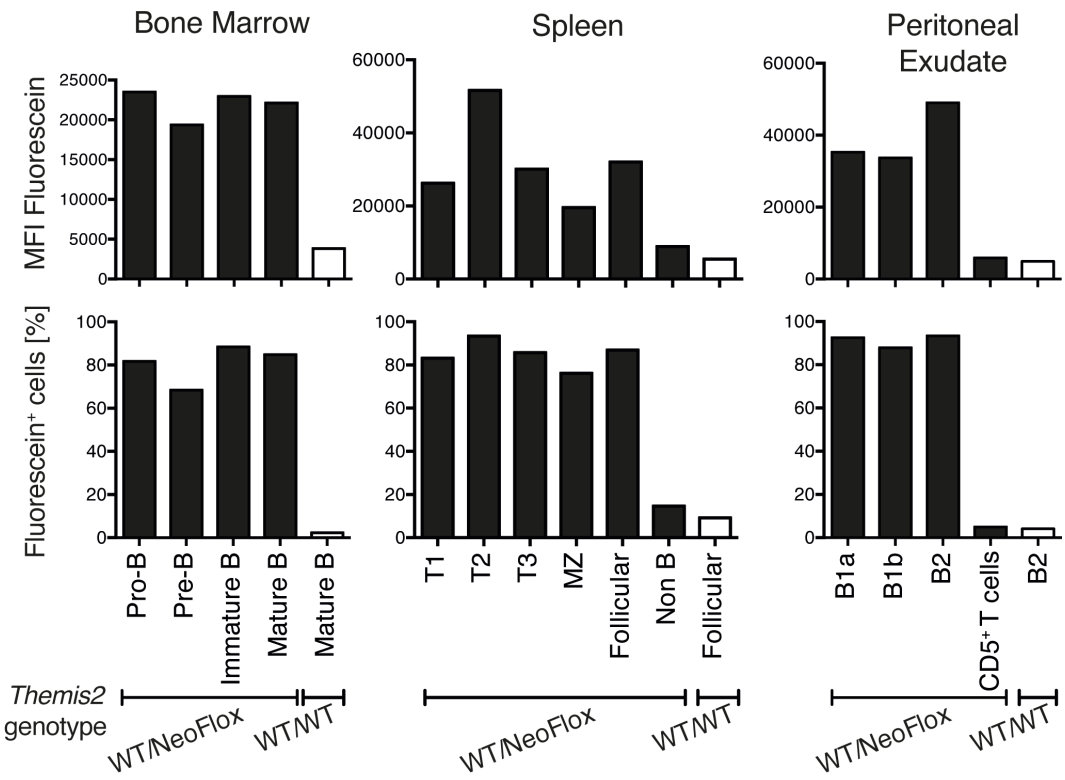

Figure 7: *Themis2* promoter activity corroborates the expression of *Themis2* in B cells.

Themis2 promoter activity was assessed by FACS-Gal as a function of the activity of the β-galactosidase reporter expressed under control of the endogenous *Themis2* promoter from the promoterless *Themis2*^{NeoFlox} allele. Fluorescein is produced from the reaction of β-galactosidase with the non-fluorescent substrate FDG. Depicted are mean fluorescence intensity (MFI) or the percentage of fluorescein positive cells among B cell developmental subsets in the bone marrow, spleen or peritoneal exudate after a 45 min reaction time stopped by addition of the inhibitor PETG. Data are representative of 3 independent experiments. MZ, marginal zone; Non B, B220^{-} cells.

3.2.3 *Themis2* splice variants

Several splice variants were initially reported for human THEMIS2 (Treeck et al., 2002) whereas so far in public databases only a single isoform has been annotated in mice. I sought to determine whether there were different splice variants in murine B cells. Therefore I designed PCRs with primers located in exon 1 and exon 6 of murine *Themis2* to test whether any exons might be skipped and amplicons shorter than expected for *Themis2* mRNA containing all six exons (full-length *Themis2*) would be produced in the reactions. Four different primer pairs used in gradient PCRs to allow for various degrees of stringency in primer annealing, showed that full-length *Themis2* was the only mRNA species containing both exon 1 and exon 6 (Figure 8). Unspecific bands in between different reactions were inconsistent in their expected, relative molecular lengths, generally more prone to changes in annealing temperature and consequently did not support the idea of low amounts of shorter *Themis2* transcripts but rather off-target amplifications. This suggests that full-length *Themis2* is the only isoform, at least in follicular B cells, as no additional transcriptional start or termination sites have been reported for murine *Themis2* to date.

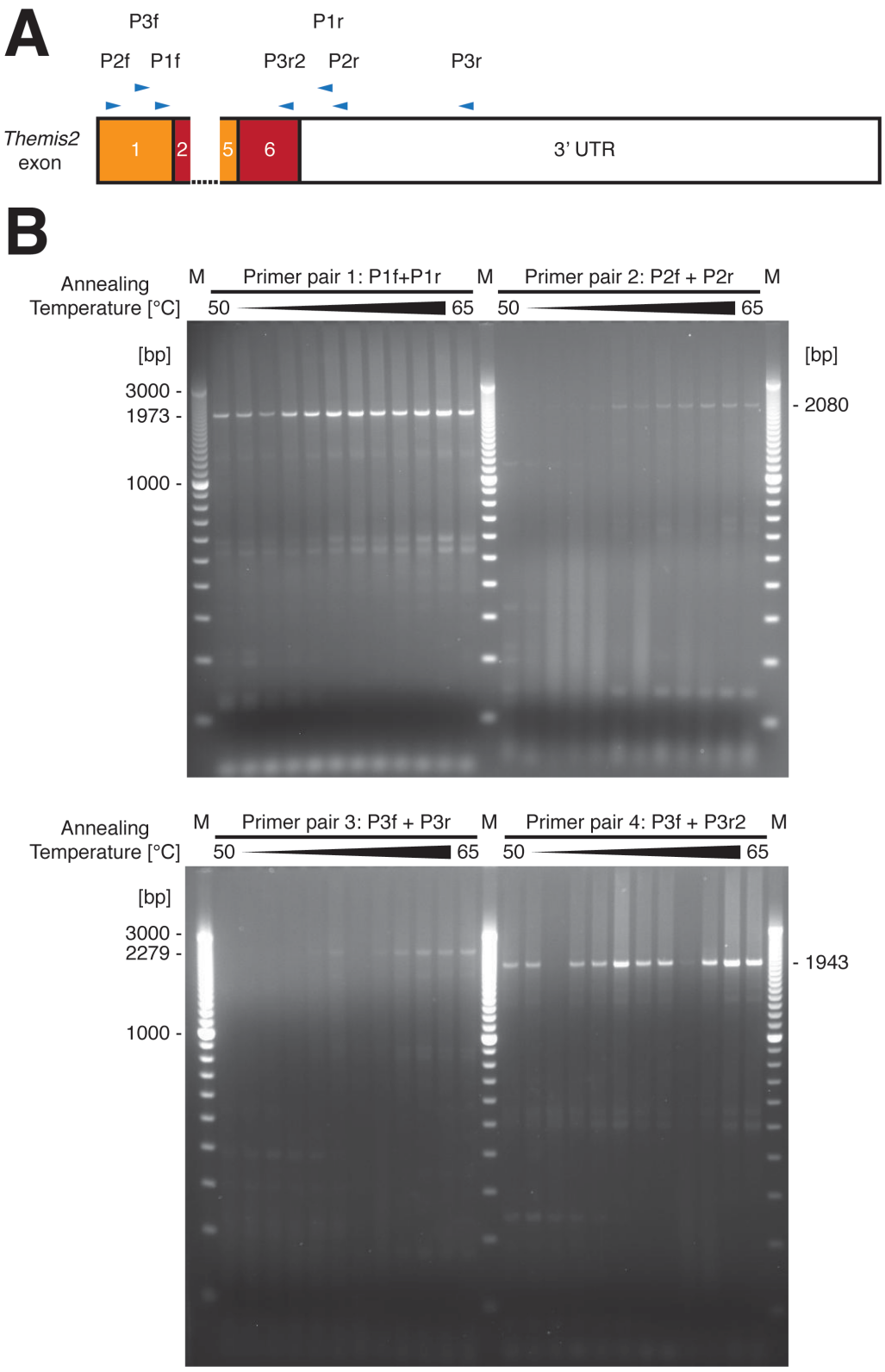

Figure 8: A transcript comprised of all six exons is the major splice variant of *Themis2* in follicular B cells.

(A) Cartoon of relevant exons indicating primer locations and **(B)** agarose gels from four different PCRs with cDNA from total RNA of follicular B cells

as template and amplified with four different primer pairs. Forward primers of each pair anneal in *Themis2* exon 1 and reverse primers in exon 6 CDS or 3' UTR. To avoid unspecific amplifications, the stringency of the PCR reaction was varied by an annealing temperature gradient from 50 – 65 °C as indicated by the black triangles. PCR reactions were resolved in a 2 % agarose gel. Expected band sizes for full-length *Themis2* mRNA: Primer pair 1: 1973 bp; Primer pair 2: 2080 bp; Primer pair 3: 2279 bp; Primer pair 4: 1943 bp; M, 100-bp DNA ladder (30 bands, 100 – 3000 bp).

3.3 Analysis of *Themis2* expression in *Themis2*-deficient mice

In order to analyse the function of *Themis2* in B cells, I created *Themis2*-deficient mice with the *Themis2*^{KO} allele. *Themis2*$^{KO/KO}$ mice were viable, bred in normal numbers and were produced at expected Mendelian ratios from heterozygous parents as shown in Supplementary Figure 7.

3.3.1.1 Analysis of Themis2 splicing in Themis2-deficient B Cells

To analyse whether *Themis2*$^{KO/KO}$ mice are truly deficient for *Themis2*, I performed qRT-PCR analysis of *Themis2* mRNA at various exon junctions (Figure 9A). I found that in *Themis2*$^{KO/KO}$ mice *Themis2* mRNA is expressed at normal levels compared to wild type controls but that exon 4 is missing as predicted. Instead, a new splice junction is created and the message is spliced from exon 3 into exon 5 in *Themis2*$^{KO/KO}$ B cells whereas such splicing was not detectable in wild type B cells (Figure 9B). These data were confirmed by RNA sequencing analysis. In *Themis2*$^{KO/KO}$ follicular B cells no reads align to *Themis2* exon 4 indicating its deletion, instead reads aligning over an exon 3 - 5 splice junction are readily detectable (Figure 9C). This leads to the predicted frameshift of exon 5 and 6 compared to exon 3 as confirmed by the RNA sequencing (Figure 9D). As the first premature stop codon caused by the frameshift is only found in exon 6, the last exon, it is not surprising that the mRNA is not

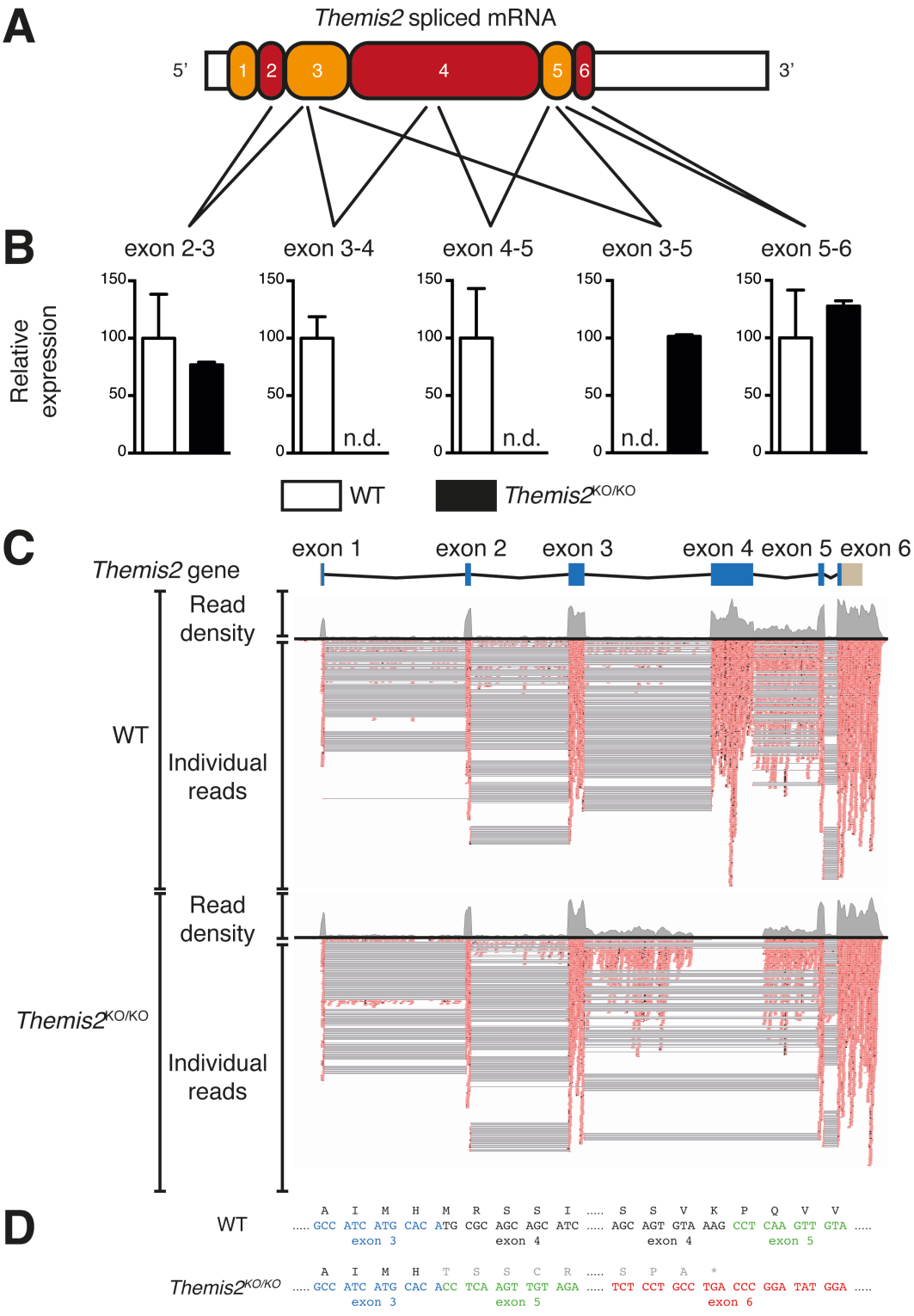

Figure 9: *Themis2* exon 4 is deleted in *Themis2*$^{KO/KO}$ mice leading to a truncated and frameshifted transcript and a novel splice junction.

(A) Cartoon indicating *Themis2* mRNA exon junctions and **(B)** qRT-PCR analysis of the expression of Themis2 across the indicated exon junctions in splenic B cells from *Themis2*$^{KO/KO}$ mice or wild type (WT) littermate

controls. WT was set to 100. n.d. not detected. Data are displayed as mean ± SEM of a least 2 biological replicates. **(C)** Alignment of RNAseq data from wild-type (WT) and *Themis2*$^{KO/KO}$ follicular B cells to the *Themis2* gene, showing loss of *Themis2* exon 4 and splicing from exon 3 to exon 5 in *Themis2*$^{KO/KO}$ cells. Light brown: UTR; blue: coding sequence; grey profile plot: maximum read density; stacked red boxes: individual reads; black lines: connection between two parts of a split read showing splicing. **(D)** RNAseq-derived sequence of relevant *Themis2* mRNA exons and predicted translation showing that exon 3 is spliced directly to exon 5 giving rise to a frameshift in translation (light gray font) and ending in a stop codon (*) in exon 6.

degraded by nonsense mediated RNA decay as this process requires a premature stop codon in an exon that is not the final 3' exon (Schweingruber et al., 2013). Furthermore, the RNA sequencing data confirm the previous PCR results suggesting that full-length *Themis2* transcript containing all six exons is the only splice variant in murine follicular B cells.

3.3.2 THEMIS2 protein expression

To confirm the absence of full-length THEMIS2 protein I performed immunoblots. I found four different antibodies against murine THEMIS2 giving bands at the expected molecular weight of about 74 kDa. The epitopes of the antibodies are located in exon 4 and exon 6 (Figure 10A). Immunoblots probed with four antibodies show that no full-length THEMIS2 protein is detectable in cell extracts from *Themis2*$^{KO/KO}$ splenic B cells (Figure 10B and 10C). Immunoblots probed with an antibody specific for an epitope in exon 6, did not show any new, smaller additional bands, consistent with the prediction that if any truncated THEMIS2 was produced in *Themis2*$^{KO/KO}$ B cells, its sequence is frameshifted, resulting in a different amino acid sequence and termination before the epitope in exon 6. Epitopes in exon 4 also vanish due to its genomic deletion. Unfortunately, in the absence of a working N-terminal anti-THEMIS2 antibody I was unable to determine whether a truncated THEMIS2 protein, potentially consisting of the N-terminal part of CABIT1 and a frameshifted tail, was produced and stable in *Themis2*$^{KO/KO}$ mice.

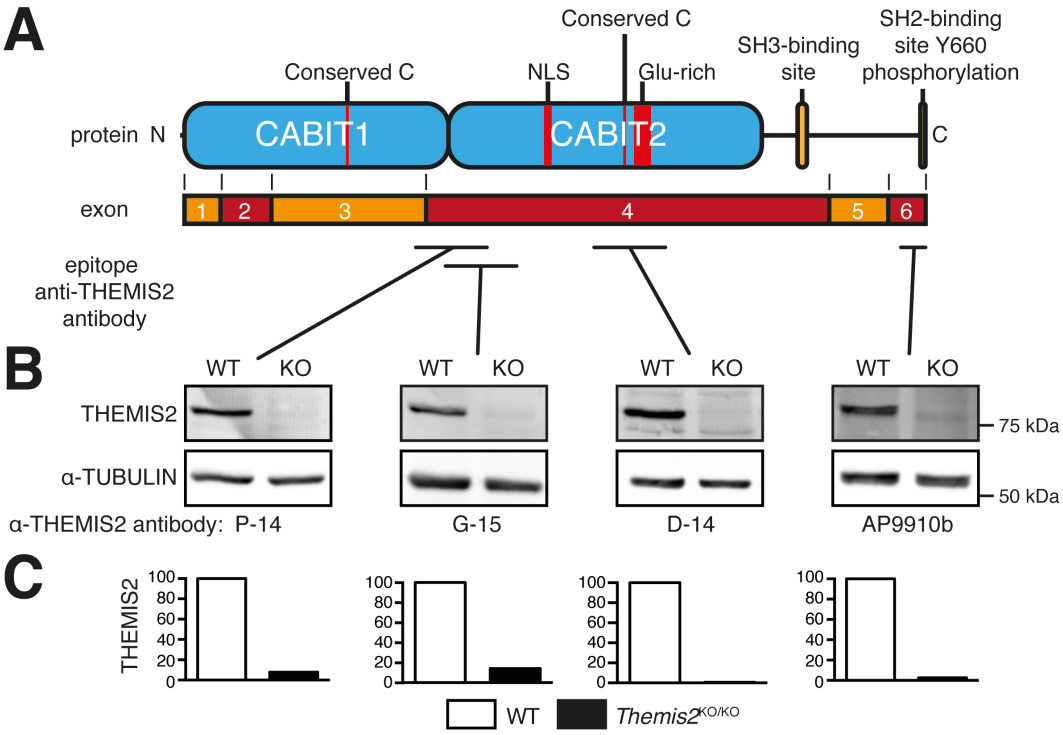

Figure 10: **Full-length THEMIS2 protein is not expressed in *Themis2*$^{KO/KO}$ splenic B cells.**

(A) Cartoon representing murine THEMIS2 protein domains, their encodement by exons and localisation of epitopes of used anti-THEMIS2 antibodies. **(B)** Immunoblot analysis of THEMIS2 protein expression in splenic B cells from wild type (WT) or *Themis2*$^{KO/KO}$ (KO) mice using four different anti-THEMIS2 antibodies with the indicated epitopes localised in either exon 4 or exon 6. **(C)** Quantification of (B), THEMIS2 bands were normalised to α-TUBULIN bands, WT was set to 100. Data are representative of 2 to 3 biological replicates in independent experiments.

3.4 Analysis of B cell development in *Themis2*-deficient mice

Having confirmed the absence of full-length THEMIS2 in *Themis2*$^{KO/KO}$ mice I began their phenotypic analysis. As mice deficient for the T cell-restricted gene *Themis1* show a strong impairment in T cell development, I first focussed on the analysis of B cell development in *Themis2*$^{KO/KO}$ mice.

3.4.1 B cell development in *Themis2*-deficient mice

I analysed the numbers of developing, mature and antigen-experienced B cells subset under steady-state conditions by flow cytometry in various lymphoid organs including the bone marrow, spleen, peritoneal exudate, peripheral and mesenteric lymph nodes, Peyer's patches and blood. The analysis included developing pro-B, pre-B, immature and mature recirculating B cells in the bone marrow, developing transitional type 1, 2 and 3 cells, as well as follicular and marginal zone B cells in the spleen and B1a, B1b and B2 B cells in the peritoneal cavity. I also quantified the numbers of antigen-experienced cells counting plasma cells in the bone marrow and spleen as well as germinal centre B cells, plasmablasts and regulatory B cells in the spleen. The number of all analysed subsets was unchanged in *Themis2*$^{KO/KO}$ mice compared to wild type controls (Figure 11). Cell surface marker expression on *Themis2*$^{KO/KO}$ cells was also identical to control animals (Figure 12). I conclude that by evaluation of cell surface markers the development of B cells from early progenitor

cells such as pro-B cells to fully differentiated cells such as plasma cells proceeds unperturbed in a *Themis2*-deficient context. As expected, the numbers of $CD4^+$ and $CD8^+$ T cells is also normal in *Themis2*$^{KO/KO}$ mice.

3.4.2 B cell development of *Themis2*-deficient bone marrow in competition with wild type bone marrow in mixed bone marrow chimeras

As B cell development was normal in mice constitutively deficient for *Themis2*, I decided to use a more sensitive approach by making mixed bone marrow radiation chimeras. In these chimeras *Themis2*-deficient cells are in competition with wild type cells making defects much more apparent. Either *Themis2*$^{KO/KO}$ or wild type $CD45.2^+$ bone marrow together with wild type $CD45.1^+$ bone marrow was injected into sublethally irradiated *Rag1*-deficient mice to test whether *Themis2*-deficient cells had a developmental defect.

I created chimeras at four different ratios of $CD45.2^+/CD45.1^+$ bone marrow cells (100 %, 50 %, 20 % or 10 % $CD45.2^+$ cells) (Figure 13). Under each of these conditions, *Themis2*-deficient bone marrow performed equally well at creating the indicated B cell populations in the bone marrow, spleen and lymph nodes. Pro-B cell numbers are slightly higher than the injection ratio as some of these cells derive from the $CD45.2^+$ *Rag1*-deficient bone marrow since development is blocked after the pro-B cell stage in these mutants (Mombaerts et al., 1992).

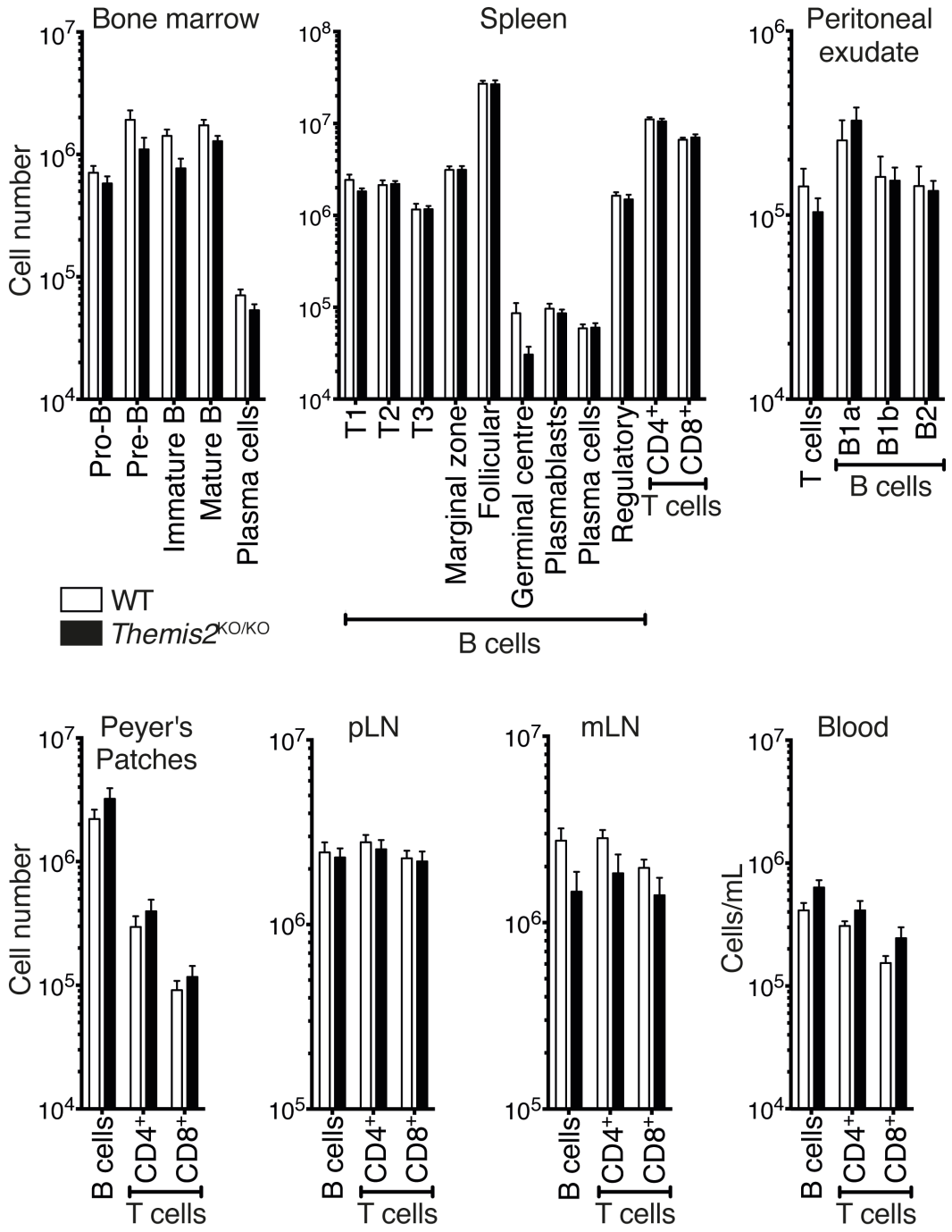

Figure 11: B cells numbers are normal in *Themis2*$^{KO/KO}$ mice.

Numbers of B and T cell populations in the indicated organs of *Themis2*$^{KO/KO}$ and WT mice analysed by flow cytometry. pLN, peripheral lymph nodes; mLN, mesenteric lymph nodes. Cell populations were gated as shown in Figure 12. Bars represent mean ± SEM of 8 biological replicates in 3 independent experiments.

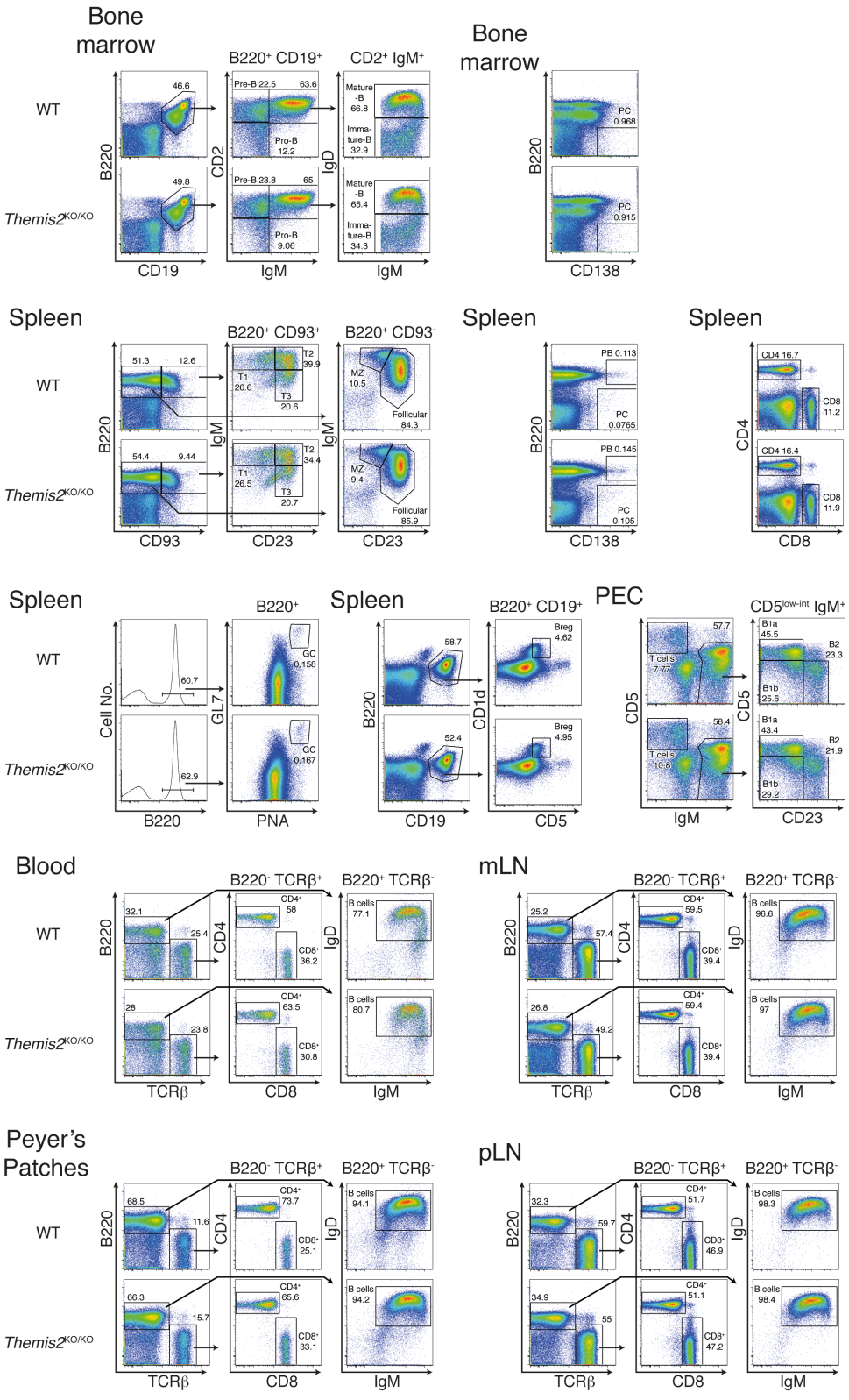

Figure 12: *Themis2*$^{KO/KO}$ mice show normal B cell development and B cell lineage surface marker expression.

Flow cytometric surface marker expression and gating strategy used for cell population analysis in Figures 11 and 13 and for cell sorting in

Figure 6. Names next to gates indicate the population; numbers indicate the percentage of cells within the marked gate on the total plot. Gating strategies were as follows: in bone marrow, pro-B cells ($B220^+$ $CD19^+$ $CD2^-$ IgM^-), pre-B cells ($B220^+$ $CD19^+$ $CD2^+$ IgM^-), immature B cells ($B220^+$ $CD19^+$ $CD2^+$ IgM^+ IgD^-), mature B cells ($B220^+$ $CD19^+$ $CD2^+$ IgM^+ IgD^+); in spleen, transitional type 1 (T1, $B220^+$ $CD93^+$ $CD23^-$ IgM^+), type 2 (T2, $B220^+$ $CD93^+$ $CD23^+$ IgM^+) and type 3 (T3, $B220^+$ $CD93^+$ $CD23^+$ IgM^{low}) B cells, marginal zone B cells (MZ, $B220^+$ $CD93^-$ $CD23^-$ IgM^+) follicular B cells ($B220^+$ $CD93^-$ $CD23^+$ IgM^+), plasmablasts (PB, $B220^+$ $CD138^+$) plasma cells (PC, $B220^-$ $CD138^+$), germinal centre B cells (GC, $B220^+$ PNA^+ $GL7^+$) and regulatory B cells (Breg, $B220^+$ $CD19^+$ $CD1d^+$ $CD5^+$), CD4 T cells ($CD4^+$), CD8 T cells ($CD8^+$); in peritoneal exudate cells (PEC), B1a cells (IgM^+ $CD5^+$ $CD23^-$), B1b cells (IgM^+ $CD5^-$ $CD23^-$) and B2 cells (IgM^+ $CD5^-$ $CD23^+$), T cells ($CD5^+$ IgM^-); blood, mesenteric lymph nodes (mLN), peripheral lymph nodes (pLN) and Peyer's patches, B cells ($B220^+$ $TCR\beta^-$ IgM^+ IgD^+), CD4 T cells ($B220^-$ $TCR\beta^+$ $CD4^+$ $CD8^-$), CD8 T cells ($B220^-$ $TCR\beta^+$ $CD4^-$ $CD8^+$).

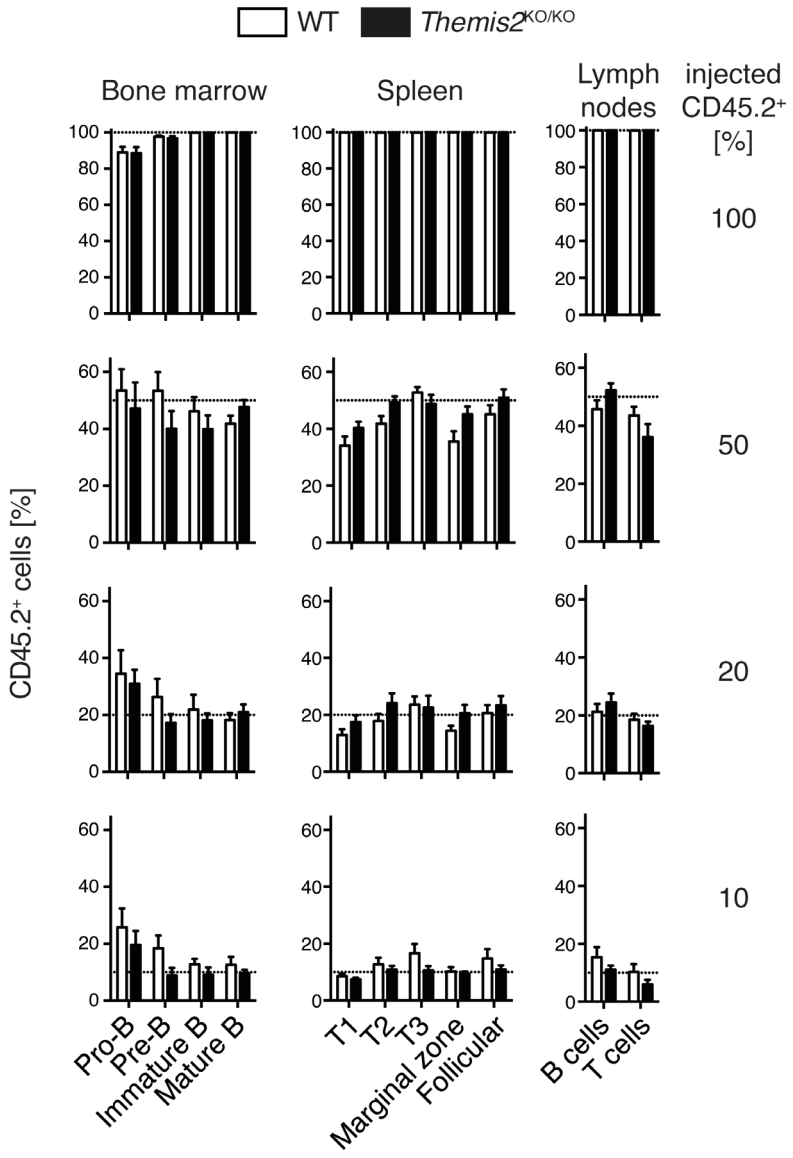

Figure 13: *Themis2*-deficient bone marrow gives rise to expected B cell lineage proportions under competition from wild type bone marrow.

Bone marrow from CD45.2$^+$ *Themis2*$^{KO/KO}$ mice or wild type (WT) littermate controls was mixed with bone marrow from CD45.1$^+$ wild type mice (10, 20, 50 or 100 % CD45.2$^+$ cells) and injected intravenously into sublethally irradiated Rag1-deficient mice. After 6 weeks mice were analysed for reconstitution of the B cell lineage by flow cytometry. Cell numbers in populations were evaluated using gating strategies as in Figure 12 with additional staining for CD45.1 and CD45.2. Depicted are the mean ± SEM of 6 mice/group. Dotted lines indicate CD45.2$^+$ / CD45.1$^+$ ratio at injection.

3.4.3 *Themis2*-deficient follicular B cells are transcriptionally similar to wild type controls

Since *Themis2*-deficient B cells develop normally according to the immunophenotypic analysis, I investigated whether their transcriptome might be changed due to subtle alterations caused by the absence of *Themis2*. Therefore I analysed transcription in splenic, follicular B cells from *Themis2*$^{KO/KO}$ animals and wild type littermate controls by high throughput RNA sequencing of poly-A^{+} enriched total RNA. The results in Figure 14 show that the transcriptome of *Themis2*-deficient follicular B cells is highly similar to their wild type counterparts. Statistical analysis of differentially expressed genes resulted in only three genes being statistically significantly differentially regulated with a >2-fold change in expression between genotypes. The statistically significantly differentially regulated genes did not suggest any obvious pathways being deregulated in *Themis2*-deficient follicular B cells, neither by manually perusing the list nor by computational pathway analysis. A comprehensive gene list from this analysis is given in the appendix in section 6.3.2.

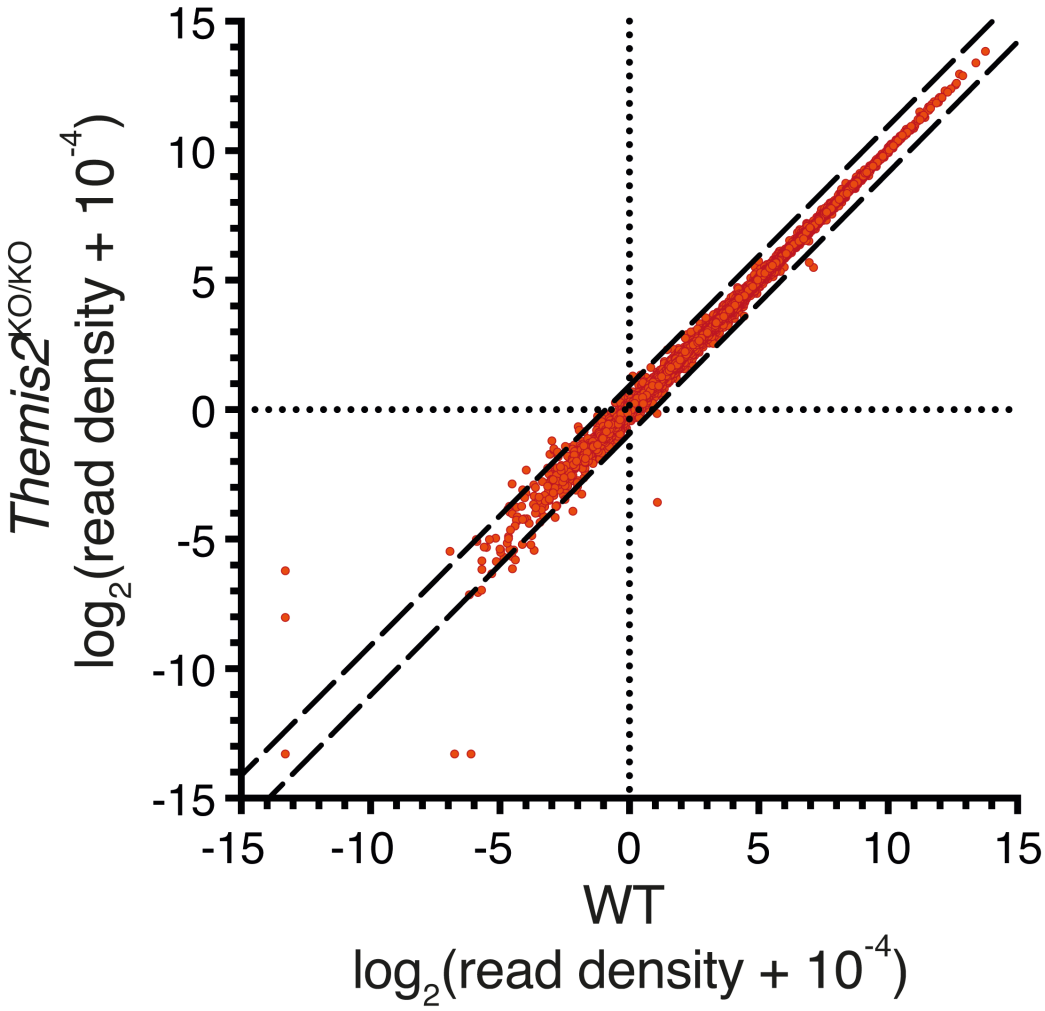

Figure 14: ***Themis2*$^{KO/KO}$ follicular B cells are transcriptionally similar to wild type controls.**

Scatterplot of gene expression of follicular B cells from *Themis2*$^{KO/KO}$ mice or wild type littermate controls sorted by flow cytometry and analysed by RNA sequencing. Expression is plotted as read densities. Each red dot represents a gene, dotted lines indicate read density = 1 and dashed lines indicate 2-fold deregulation thresholds. A value of 10^{-4} was added to the read density values to display genes with nRPKM = 0. Dots represent mean of 3 biological replicates per genotype.

3.4.4 *Themis2*-deficient follicular B cells show similar usage of immunoglobulin genes

To extend the analysis of the RNA sequencing data and check whether there are indications of differences in B cell repertoire between *Themis2*$^{KO/KO}$ and wild type control follicular B cells I analysed the usage of the immunoglobulin V, D, J and C regions at the IgH and both IgL loci. This could indicate changes in positive or negative selection. Presence of annotation for each of these genes was checked manually to verify a complete analysis of VDJ gene usage. I show that usage of V, D, J and C regions in all three immunoglobulin loci (heavy chain, κ chain and λ chain loci) are indistinguishable (Figure 15). The results suggest that the B cell repertoire is largely identical in *Themis2*-deficient mice as positive and negative selection seem to proceed normally and a bias in VDJ gene usage was not detected.

In summary, the data from the immunophenotypic analysis of constitutively *Themis2*-deficient mice and mixed bone marrow radiation chimeras as well as the genome-wide RNA sequencing analysis indicate that B cell development proceeds normally in *Themis2*$^{KO/KO}$ mice.

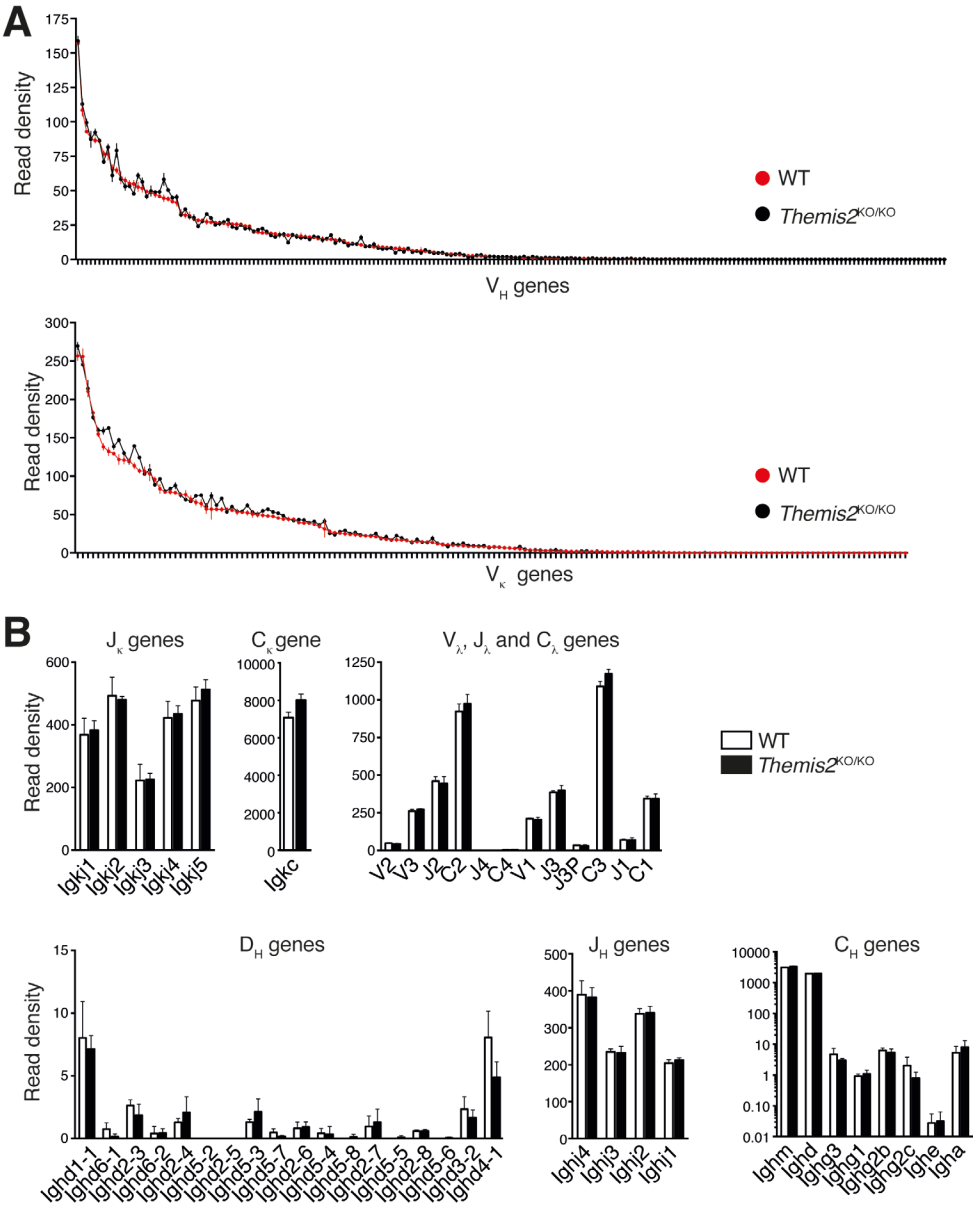

Figure 15: Normal usage of immunoglobulin genes in *Themis2*$^{KO/KO}$ follicular B cells.

(A) Expression of V_H or V_κ genes in sorted, splenic, follicular B cells from mice of the indicated genotypes as determined by RNAseq and displayed as read densities. V_H and V_κ genes are ordered by level expression in wild type (WT) mice. Data is mean ± SEM of 3 biological replicates. **(B)** Expression of J_κ, C_κ, V_λ, J_λ, C_λ, D_H, J_H and C_H genes in sorted, splenic, follicular B cells from mice of the indicated genotypes as determined by RNAseq and displayed as read densities. Data is mean ± SEM of 3 biological replicates.

3.5 Analysis of B cell activation in *Themis2*-deficient mice

Since B cell development seemed unaffected by loss of *Themis2* I proceeded to analyse B cell activation. Although according to the developmental analysis steady state numbers of antigen-experienced B cells are normal, there could be defects with respect to timing, strength or robustness of the immune response. Firstly though, in order enable correct data interpretation, I needed to test whether in activated B cells expression of other CABIT domain containing proteins is induced to exclude functional redundancy.

3.5.1 Regulation of *Themis2* expression and CABIT domain-containing proteins upon activation

To this end, I stimulated splenic B cells *in vitro* with anti-IgM, LPS or CD40L and IL-4 over a 24 h time course and analysed the expression of Themis family members via qRT-PCR at the indicated time points. Figure 16A shows that *Themis2* transcription is reduced upon *in vitro* culture but that stimulation with any of the agonists leads to an up to 40-fold greater reduction in *Themis2* mRNA. I found lowest expression at 6 h after stimulation, after which time, *Themis2* mRNA levels increased again. Other Themis family members were just above the reliable, lower limit of detection or below detection at all time points and either did not increase or further decreased during the time course. *Themis3* was not detectable at any given time point of any stimulation. The data demonstrate that

Themis2 is the only expressed Themis family member in activated splenic B cells.

To rule out any abnormal deregulation of CABIT domain containing proteins in *Themis2*$^{KO/KO}$ B cells as well as in wild type B cells I used RNA sequencing data of either follicular B cells left unstimulated or stimulated for 6 h with anti-IgM, LPS or CD40L and IL-4. Shown in Figure 16B, RNA sequencing data for wild type controls gave the same results as the qRT-PCR. Analysis of *Themis2*-deficient follicular B cells revealed that there was no abnormal upregulation of other CABIT containing proteins either. To summarise, I found no evidence of possible functional compensation of *Themis2* in activated B cells by other CABIT domain containing proteins, either in wild type or in *Themis2*-deficient cells.

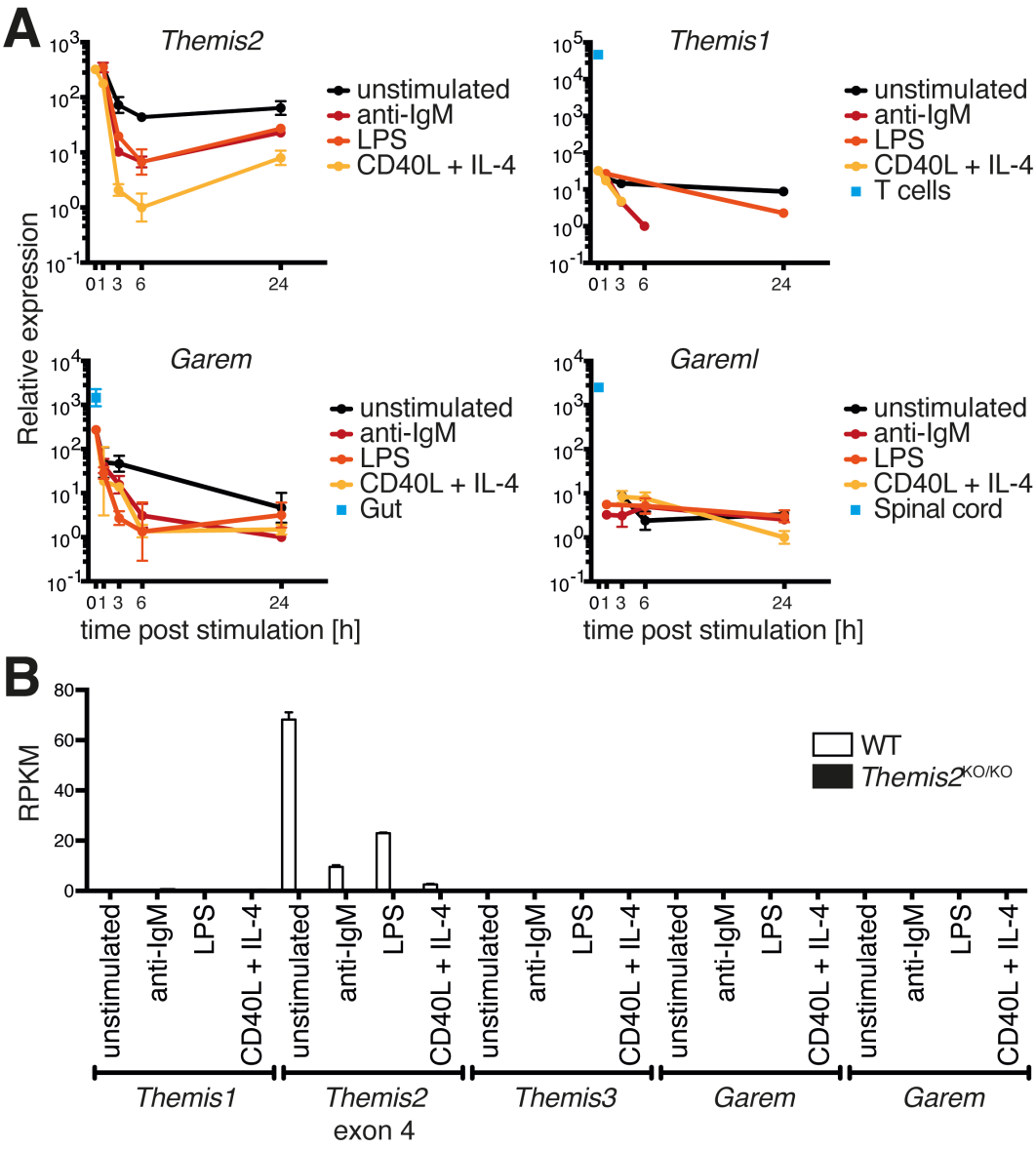

Figure 16: *Themis2* is downregulated after *in vitro* B cell activation and is expressed non-redundantly in *Themis2*-deficient and wild type B cells.

(A) qRT-PCR for relative mRNA levels of CABIT domain-containing proteins of splenic B cells *in vitro* activated with either anti-IgM, LPS or CD40L and IL-4 or left unstimulated for the indicated times. Missing data points for *Themis1*, *Garem* or *Gareml* indicate that the transcript could not be detected; *Themis3* was not detected at any time point. Graphs show mean ± SEM of 3 biological replicates and are representative of 2 independent experiments. **(B)** mRNA expression determined by RNAseq of CABIT domain-containing proteins in splenic follicular B cells from WT

or *Themis2*$^{KO/KO}$ mice either unstimulated or activated by the anti-IgM, LPS or CD40L and IL-4 for 6 h. Data are displayed as mean ± SEM reads per kilobase per million reads (RPKM) of 1 – 3 biological replicates. Expression of *Themis1*, *Themis3*, *Garem* and *Gareml* was not detectable. Unstimulated WT data is from the same experiment as in Figure 6A.

3.5.2 *In vitro* B cell activation

As functional redundancy was excluded I tested several, early B cell activation events *in vitro*.

3.5.2.1 Cell surface activation markers

Firstly, I checked whether the markers of activation CD69, CD86, CD23 and MHC class II were regulated in a similar manner in *Themis2*$^{KO/KO}$ and wild type control B cells. Cells were put into culture and stimulated with titrations of either anti-IgM, LPS, BAFF, CD40L, IL-4 or CD40L with IL-4 . PMA and ionomycin were used as a control to activate B cells. I performed several titrations of the stimuli as it had been reported for *Themis1*-deficient mice that weak agonists induced stronger TCR signalling whereas stronger agonists induced identical TCR signalling compared to wild types (Fu et al., 2013). I also included stimulation with BAFF, although not a B cell activating cytokine per se, it is a well-known factor for B cell survival and activates similar signalling pathways as BCR engagement (Schweighoffer et al., 2013). I did not detect any reproducible differences in cell surface activation marker expression to any stimulus between *Themis2*-deficient B cells and wild type controls (Figure 17). This suggests that immediate events after B cell activation are normal in *Themis2*$^{KO/KO}$ B cells.

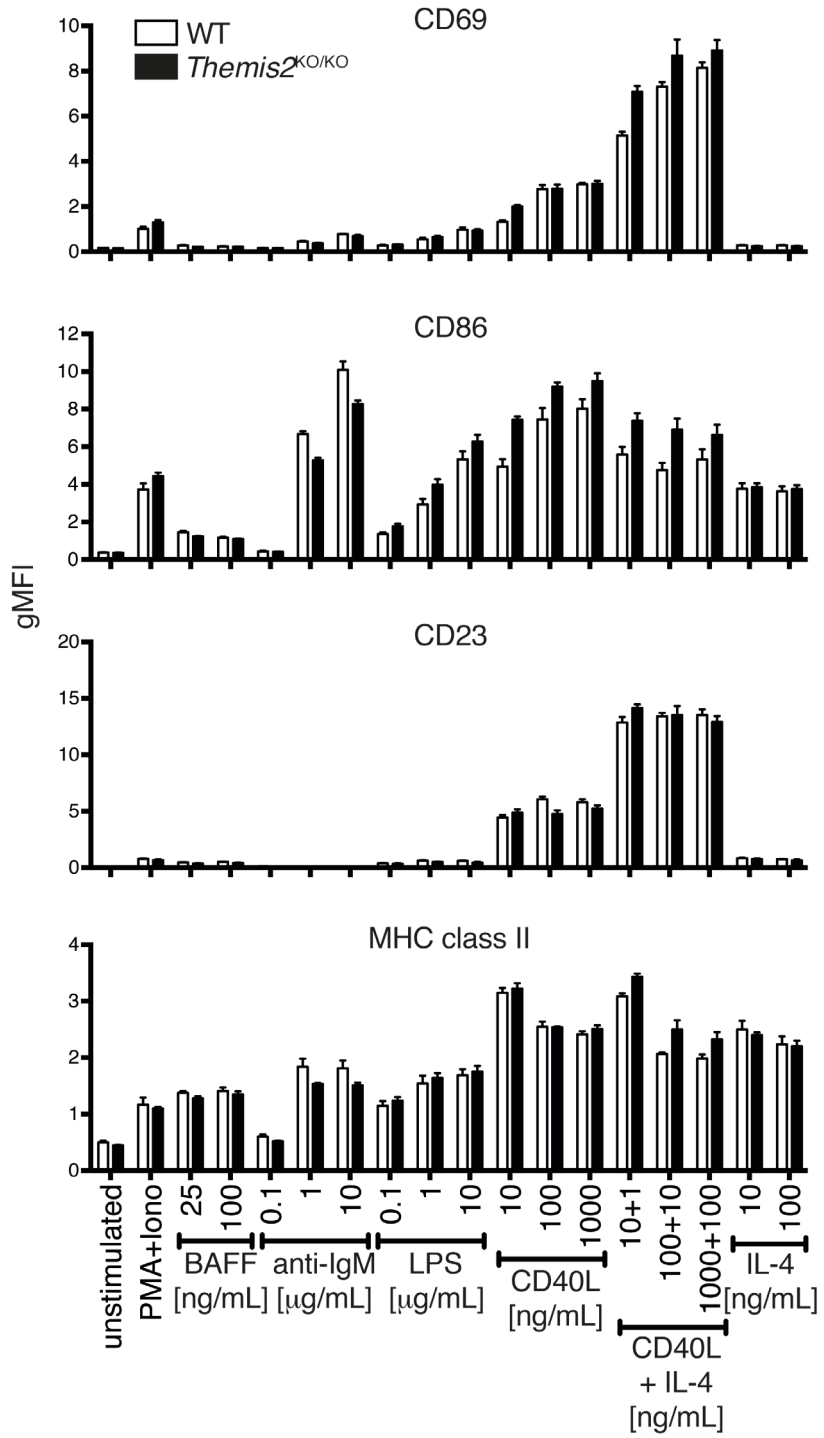

Figure 17: *Themis2*-deficient B cells show normal regulation of activation markers in response to various stimuli *in vitro*.

Geometric mean fluorescence intensity (gMFI) measured by flow cytometry of CD69, CD86, CD23 and MHC class II on splenic B cells in response to various stimuli after *in vitro* culture for 24 h. Bars indicate mean ± SEM of 3 biological replicates and is representative of 5 similar experiments.

3.5.2.2 Survival and proliferation

Using a similar approach I tested whether B cell survival or proliferation were altered in *Themis2*-deficient B cells *in vitro*. I cultured splenic B cells with the same stimuli as above and measured proliferation by CFSE dilution and live cells by amino-reactive dye stain using flow cytometry. The results shown in Figure 18 demonstrate that both proliferation and survival are identical between genotypes when stimulated with any of the six different agonists. In conclusion, B cells survival is not compromised by *Themis2*-deficiency and cells have normal proliferative capacity in response to a range of different stimuli.

3.5.2.3 Cytokine production

Since I had not observed any defects in proliferation, survival or the expression of cell surface markers, I went on to measure the expression of 16 different cytokines (CCL2/JE/MCP-1, CXCL1/KC, CXCL2/MIP-2, GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p70, IL-13, IL-17A, TNFα, VEGF-A) after *in vitro* stimulation with the above-mentioned stimulants. I found the most highly expressed cytokines to be IL-6, IL-10, TNFα, and VEGF-A (Figure 19) depending on the condition whereas other factors were hardly detectable in the B cell cultures (Supplementary Figure 8). I found neither reduced expression nor unexpected elevated expression of any of the 16 cytokines between *Themis2*$^{KO/KO}$ and wild type

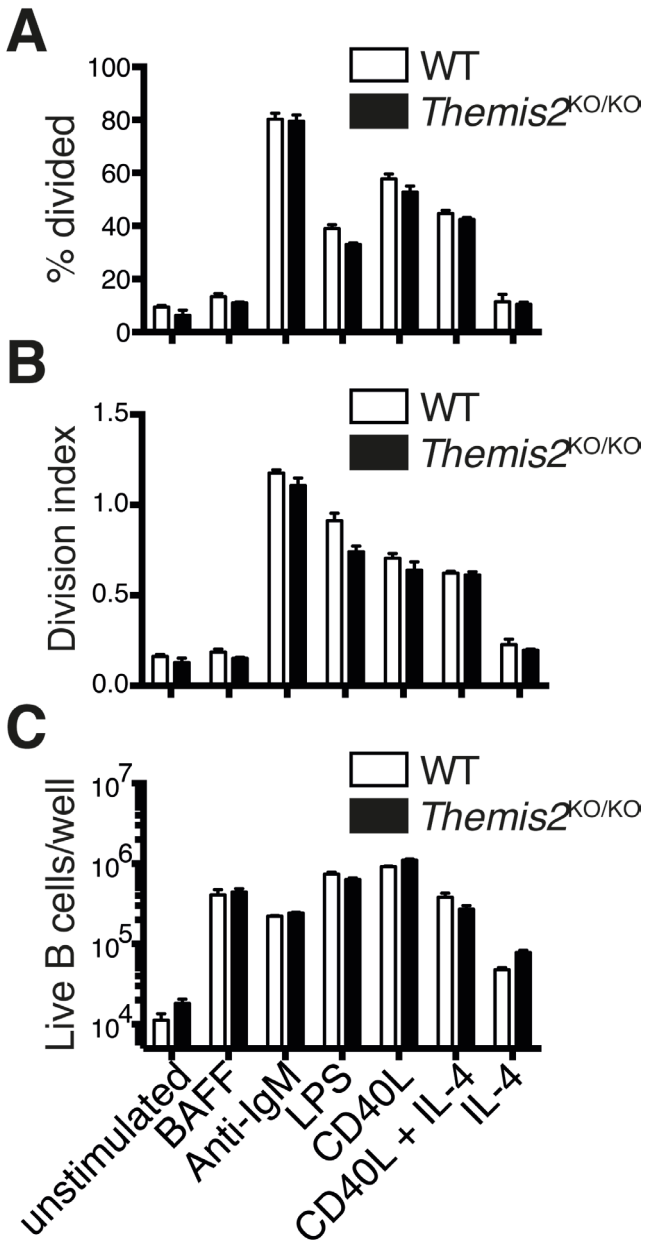

Figure 18: *In vitro* survival and proliferation are normal in *Themis2*-deficient B cells.

Splenic B cells from WT or *Themis2*$^{KO/KO}$ mice were cultured for 72 h with the indicated stimuli. Cell proliferation and survival were measured by CFSE dilution and amine reactive dye staining respectively and analysed by flow cytometry. Graphs indicate mean ± SEM of **(A)** the percentage of cells that had divided or **(B)** the division index representing the average number of divisions per cells in the assay or **(C)** the numbers of live B cells. Data shown are from 3 biological replicates and are representative of 3 similar experiments.

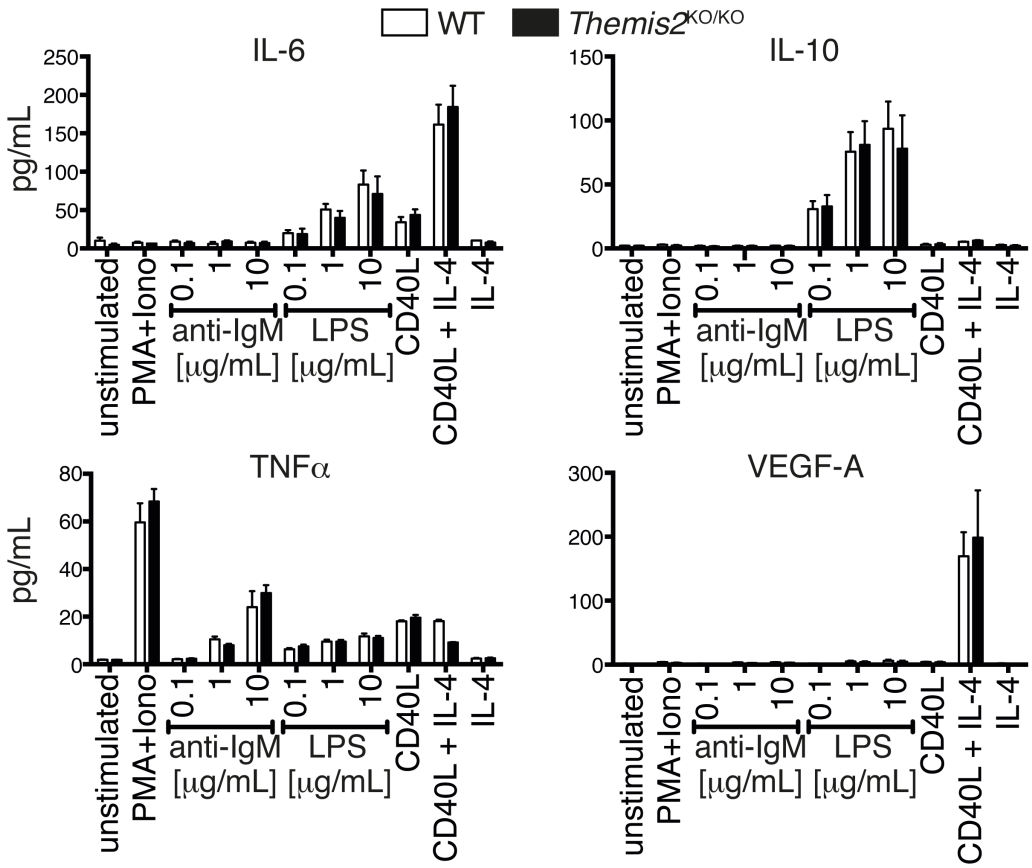

Figure 19: *Themis2*-deficient B cells produce normal amounts of cytokines upon *in vitro* activation.

Cytokines determined by Luminex 16-plex assay in the supernatant of B cells cultured for 72 h in the presence of the indicated stimuli. Graphs show mean ± SEM of 3 biological replicates and is representative of 3 independent experiments.

B cells. The results indicate that stimulation-induced cytokine and chemokine secretion are not perturbed in *Themis2*-deficient B cells.

3.5.2.4 Analysis of gene expression in activated Themis2-deficient B cells by RNA sequencing

I then performed massively parallel RNA sequencing on follicular B cells stimulated with anti-IgM or LPS or CD40L and IL-4 and found slightly more significantly differentially expressed genes (found in the appendix sections 6.3.3 to 6.3.5) compared to unstimulated samples but failed to detect any prominent outliers between wild type and *Themis2*-deficient B cells (Figure 20). Only 3, 8, and 17 genes, respectively, were differentially expressed > 2-fold between *Themis2*-deficient and wild type control B cells. Pathway analysis of all statistically significantly differentially expressed genes indicated mostly pathways that had already been analysed such as cell development and haematopoiesis. Other pathways where both the expression of the involved genes and the statistical probability of this pathway being dysregulated were low, were not pursued any further with the exception of cholesterol biosynthesis pathways, as discussed in section 3.5.2.5. I therefore concluded that follicular *Themis2*$^{KO/KO}$ B cells whether stimulated or not, were transcriptionally very similar to their wild type counterparts.

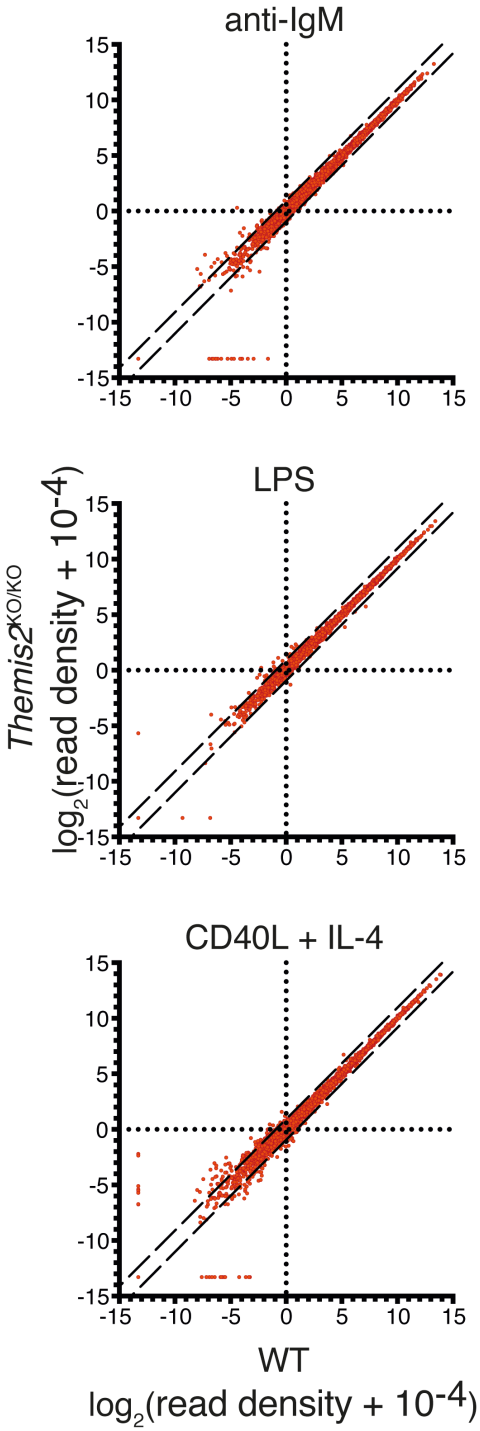

Figure 20: *In vitro* activated *Themis2*-deficient follicular B cells show similar gene expression compared to wild type controls.

Scatterplots showing comparison of gene expression measured by RNAseq in splenic B cells from *Themis2*$^{KO/KO}$ and wild type (WT) mice cultured for 6 h in the presence of anti-IgM, LPS or CD40L and IL-4. Data are displayed as in Figure 14. Dots represent mean of 1 – 3 biological replicates per genotype and condition.

3.5.2.5 BCR internalisation

Three facts prompted the testing of the capacity of *Themis2*$^{KO/KO}$ B cells to internalise the BCR after cross-linking Firstly, I found genes of cholesterol metabolism to be among the differentially regulated genes of the RNA sequencing experiment. Pathway analysis suggested that cholesterol pathways were deregulated under LPS-stimulated as well as unstimulated conditions due to reduced (1.12 - 1.52-fold) expression of *Acat2*, *Fdft1*, *Nsdhl*, *Cyp51a1*, *Dhcr24*, *Fdps*, *Msmo1*, *Sc5d* and *Sqle* in *Themis2*$^{KO/KO}$ B cells compared to controls. Although the differences were small, the cholesterol pathways ranked among the most statistically significantly regulated pathways compared to other pathways in the analysis and had low p-values in a right-tailed Fisher's exact test (Supplementary Figure 3B). BCR internalisation is linked to cholesterol levels and thus can serve as a read out (Bléry et al., 2006). Moreover, dysregulation of cholesterol genes has also been described for *Themis1*-deficient T cells. Still, any functional consequences of these changes in gene expression have remained obscure (Johnson et al., 2009).

Secondly, as THEMIS2 has so far been surmised to be a scaffolding protein in proximal BCR signalling, its role might be in very short-term BCR proximal signalling processes occurring rapidly after BCR cross-linking. A short-term, BCR proximal process that happens within minutes, such as BCR internalisation, had not been tested so far on *Themis2*-deficient B cells. As strong signalling defects were unlikely in view of my previous data on B cell activation, smaller kinetic changes in the time frame of

minutes could still occur. One such process that can be readily measured is BCR internalisation.

Thirdly, I noted that *Lat2*-deficient B cells have been reported to have a defect in BCR internalisation (Mutch et al., 2007). LAT2 is another scaffolding protein in BCR signalling, which shows little phenotype but is also phosphorylated after BCR stimulation and associates with GRB2 (Brdicka et al., 2002) and, in this regard, bears strong similarities to *Themis2*. Therefore, I tested the internalisation of IgM after cross-linking. As illustrated in Figure 21A, I found that the kinetics of BCR internalisation are identical between *Themis2*$^{KO/KO}$ and wild type B cells suggesting that very early events after BCR stimulation are also intact if *Themis2* expression is lost.

3.5.2.6 Antigen presentation

After BCR internalisation, B cells can present captured antigen to T cells. Thus I asked whether *Themis2*$^{KO/KO}$ B cells were also equally capable of presenting antigen and activating cognate T cells. Ovalbumin (OVA) was used as antigen and targeted to the BCR at varying concentrations whilst cross-linking the BCR. Then, these antigen-loaded *Themis2*$^{KO/KO}$ or wild type B cells were cultured with OVA-specific OT-II T cells and T cell proliferation and IL-2 secretion were measured. Assay conditions were optimised. T cell proliferation and IL-2 secretion were shown to be B cell-dependent and differential antigen loading was verified (Supplementary

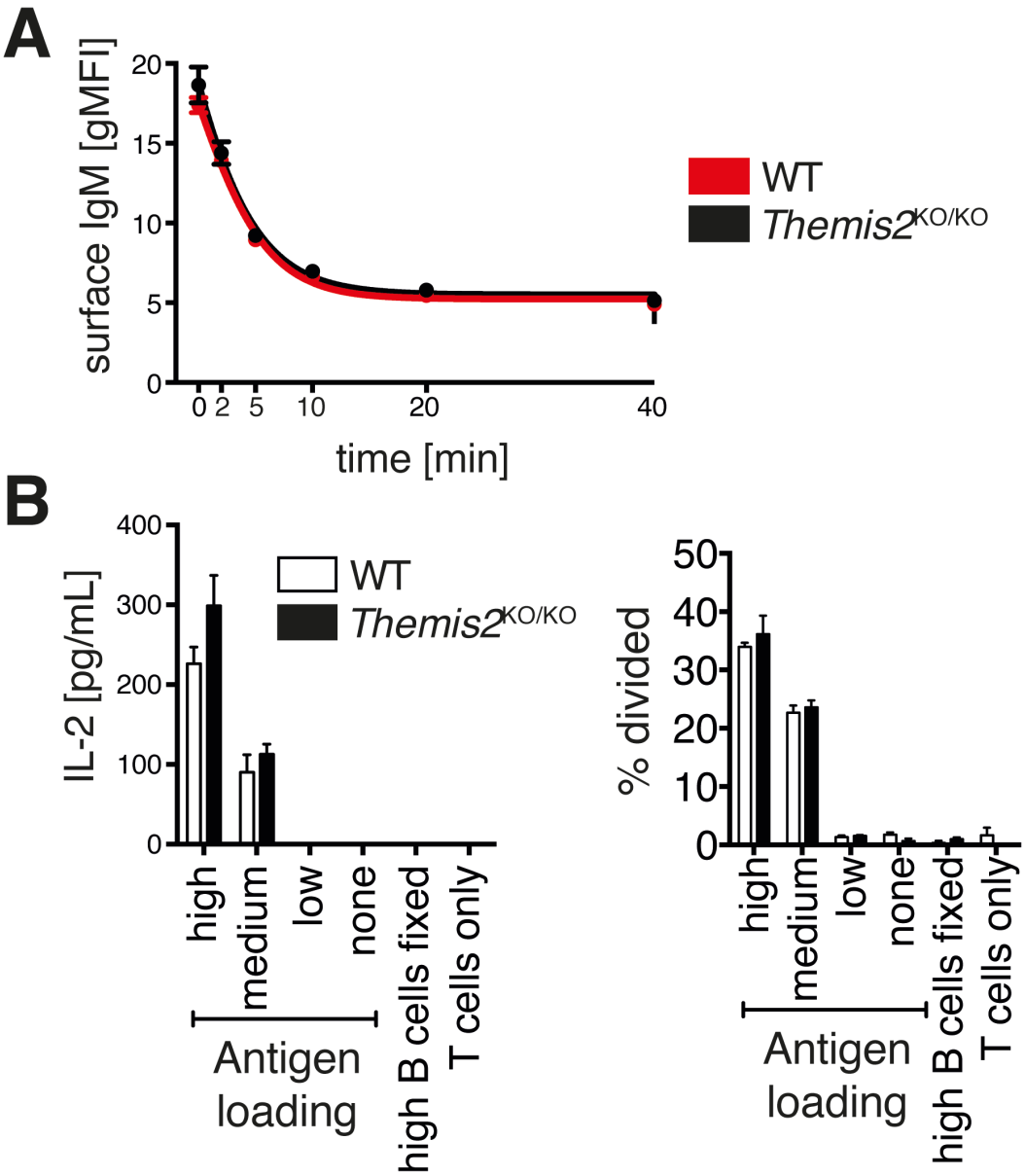

Figure 21: *Themis2*-deficient B cells show normal BCR internalisation and antigen-presentation to CD4$^+$ T cells.

(A) Kinetics of BCR surface levels on WT or *Themis2*$^{KO/KO}$ splenic B cells stimulated with anti-IgM F(ab)$_2$. Remaining surface IgM was determined by flow cytometry. Graph shows mean ± SEM geometric mean fluorescence intensity (gMFI) of 3 biological replicates and is representative of 3 independent experiments. **(B)** OT-II T cell IL-2 secretion or percentage of divided CTV-labelled OT-II T cells co-cultured for 72 h with WT or *Themis2*$^{KO/KO}$ splenic B cells that had been pre-loaded with low, medium

or high doses of ovalbumin antigen, or with no antigen. As a control OT-II T cells were also either co-cultured alone or with B cells that had been loaded with a high dose of ovalbumin antigen and were subsequently fixed with paraformaldehyde. No significant cell division or IL-2 secretion was detected under these control conditions, verifying that cytokine secretion and proliferation of T cells depends on B cell antigen internalisation and presentation. IL-2 was also not detected in the absence of T cells (Supplementary Figure 8). Bars depict mean ± SEM of 2 – 3 biological replicates and are representative of 2 independent experiments.

Figure 9). However, lack of *Themis2* does not change the ability of B cells to present antigen and activate $CD4^+$ T cells as measured by these two parameters (Figure 21B). Overall, I therefore conclude that *Themis2*-deficiency does not alter B cell activation *in vitro* or the ability of B cells to internalise and present antigen.

3.6 Analysis of antibody responses in *Themis2*-deficient mice

Next, I went on to test *in vivo* antibody responses in *Themis2*$^{KO/KO}$ mice since *in vitro* activation of B cells was normal. First, I checked whether serum antibody levels were normal in unimmunised *Themis2*$^{KO/KO}$ mice housed under SPF conditions (Figure 22). All serum immunoglobulins in C57BL6/J mice (IgM, IgG1, IgG2b, IgG2c, IgG3, IgA and IgE) were tested and showed that levels in *Themis2*$^{KO/KO}$ mice were indistinguishable from wild type littermate controls. Thus, I started the analysis of antibody responses by immunisation with various antigens.

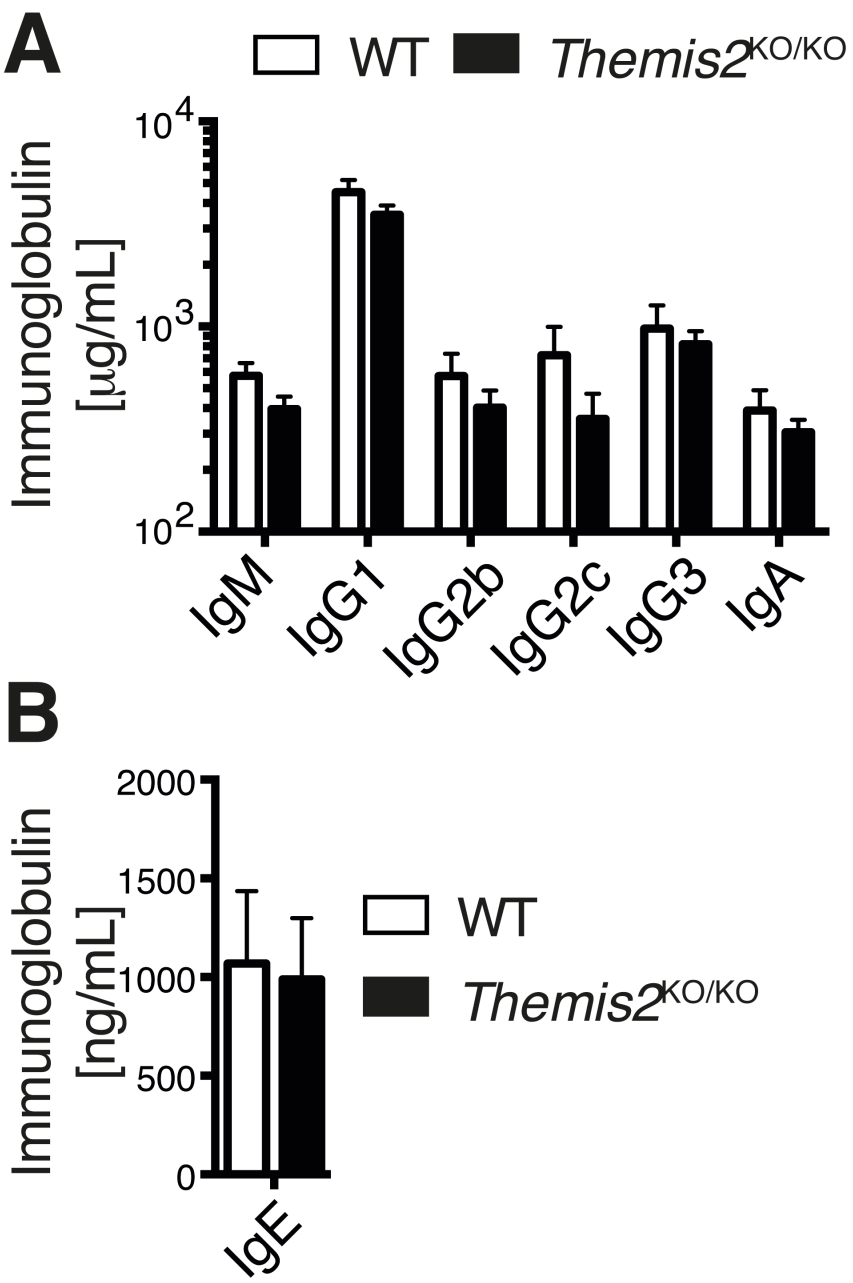

Figure 22: *Themis2*-deficient mice have normal serum immunoglobulin levels.

Levels of **(A)** IgM, IgG1, IgG2b, IgG2c, IgG3, IgA and **(B)** IgE in the serum of naïve *Themis2*$^{KO/KO}$ or wild type (WT) control mice measured by ELISA. Bars indicate mean ± SEM of 6 – 19 biological replicates and are representative of 3 independent experiments.

3.6.1 T-independent type 1 immunisation

Next, I analysed antigen-specific antibody responses to T-independent type 1 immunisation with the antigen NP-LPS. The response to NP-LPS was of particular interest as differences in response to LPS had been reported for *Themis2* overexpression and knock-down in macrophages (Peirce et al., 2010). Analysis of NP-specific IgM and IgG antibody responses up to four weeks after immunisation were identical between *Themis2*$^{KO/KO}$ mice and littermate wild type controls (Figure 23A). To get a better understanding at the cellular level, I also checked the numbers of germinal centre B cells, plasmablasts and plasma cells in the spleen five days after immunisation with NP-LPS. In agreement with the antibody data, generation of these cell types was unaltered in *Themis2*$^{KO/KO}$ mice compared to wild type controls (Figure 23B). Thus, the response to NP-LPS is normal in *Themis2*$^{KO/KO}$ mice.

3.6.2 T-independent type 2 immunisation

Next, I tested the responses of *Themis2*$^{KO/KO}$ mice to T-independent type 2 immunisation with NP-Ficoll. Anti-NP antibody titres were measured for up to four weeks but again neither NP-specific IgM nor IgG3 were altered in *Themis2*-deficient animals (Figure 24) suggesting that responses to repetitive non-protein antigens are also normal in *Themis2*-deficient mice.

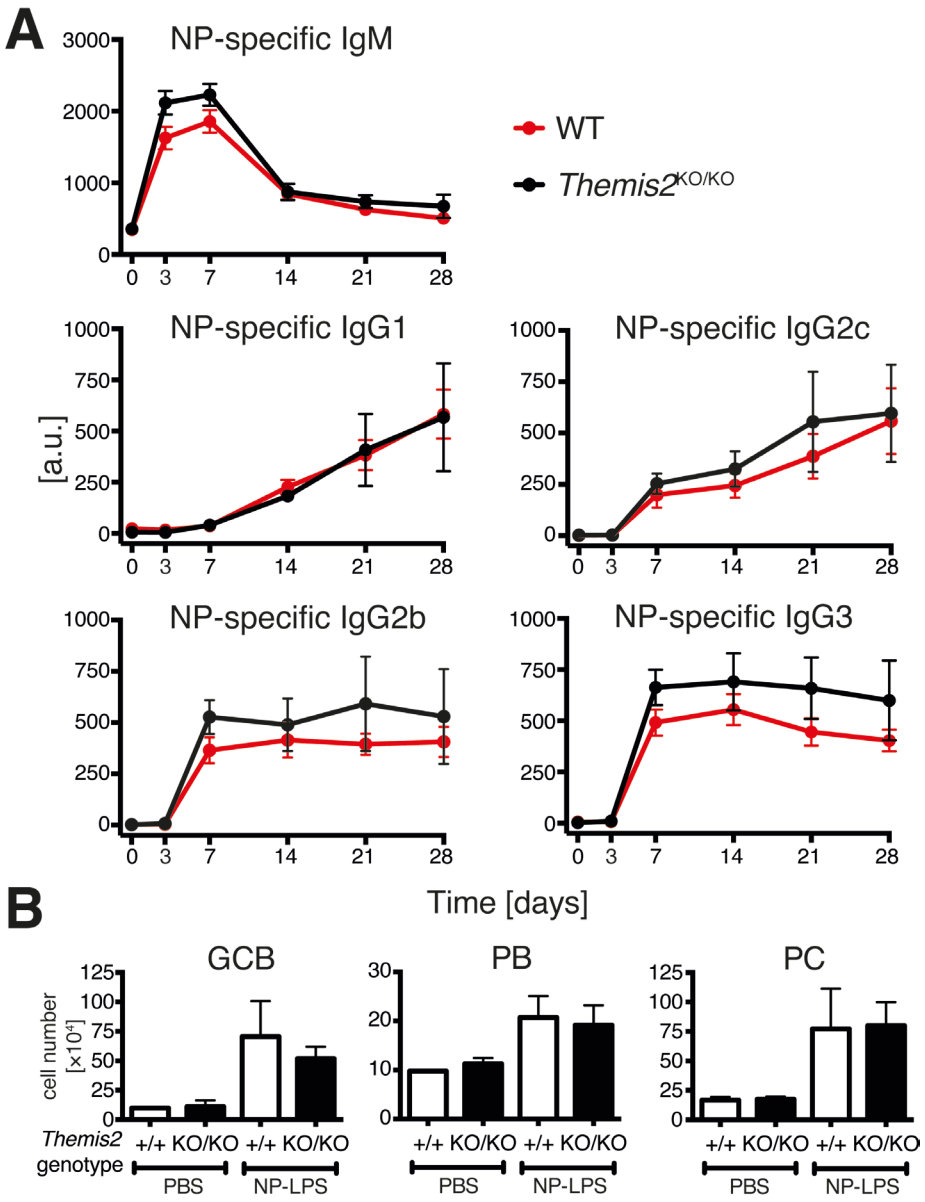

Figure 23: *Themis2*-deficient mice respond normally to immunisation with the type 1 T-independent antigen NP-LPS.

(A) Kinetics of NP-specific serum IgM, IgG1, IgG2b, IgG2c and IgG3 levels in WT and *Themis2*$^{KO/KO}$ mice following immunisation with NP-LPS on day 0 measured by ELISA. Graphs show mean ± SEM of 6 biological replicates/group and are representative of 1 – 2 independent experiments. **(B)** Absolute number of germinal centre B cells (GCB), plasmablasts (PB) or plasma cells (PC) in the spleens of *Themis2*-deficient mice on day 5 after NP-LPS immunisation. Cells were gated as in Figure 12. Bars indicate mean ± SEM and are representative of 2 (PBS) or 4 (NP-LPS) biological replicates. a.u., arbitrary units.

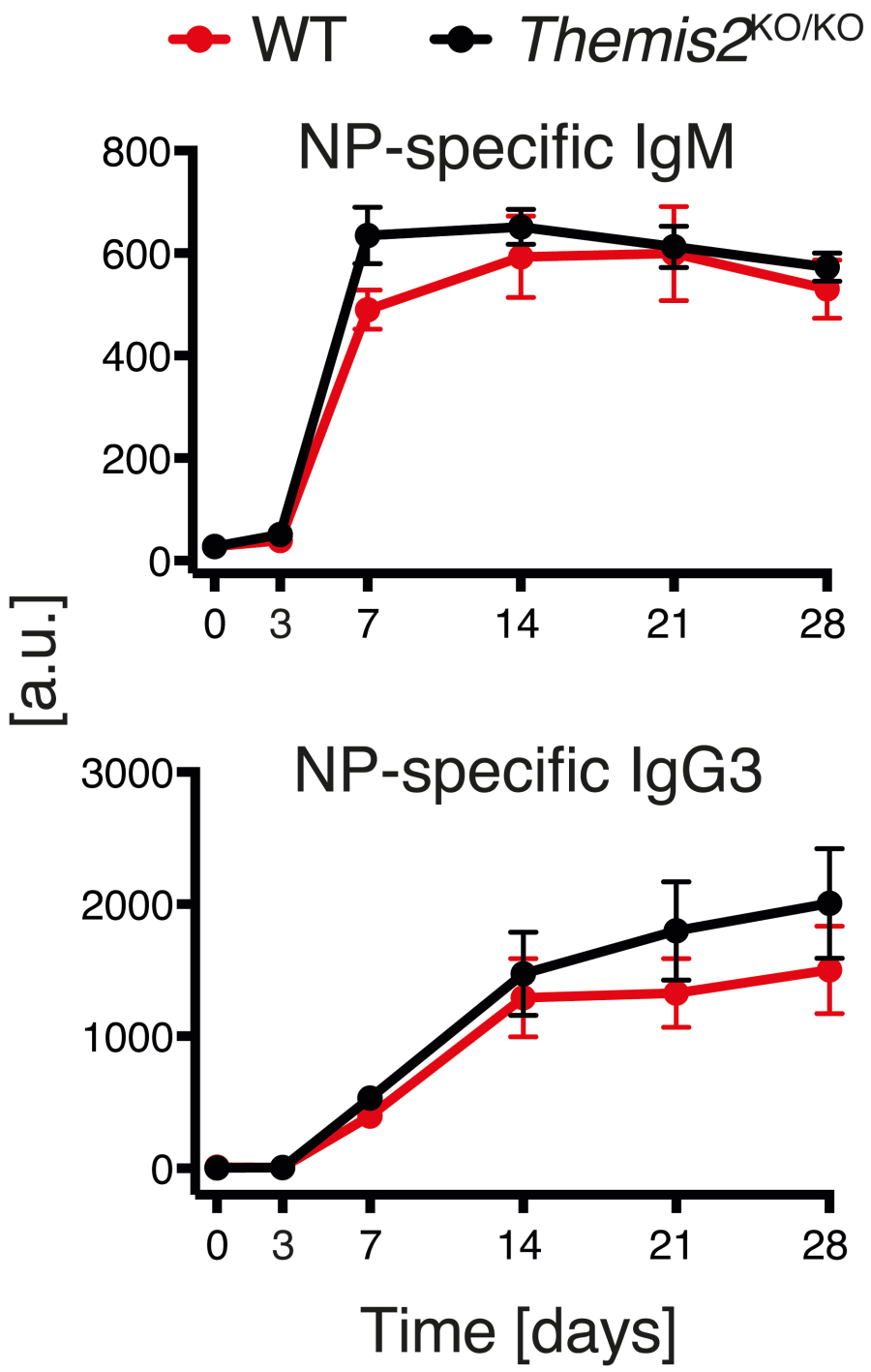

Figure 24: *Themis2*-deficient mice respond normally to immunisation with the type 2 T-independent antigen NP-Ficoll.

(A) Kinetics of NP-specific serum IgM and IgG3 in WT and *Themis2*$^{KO/KO}$ mice following immunisation with NP-Ficoll on day 0 measured by ELISA. Graphs show mean ± SEM of 6 biological replicates/group. a.u., arbitrary units.

3.6.3 T-dependent immunisations with NP-CGG and cholera toxin

Finally, I tested the ability of Themis2-deficient mice to mount antibody responses to T-dependent antigens. Immunisation of *Themis2*$^{KO/KO}$ animals or wild type controls with the T-dependent antigen NP-CGG in alum resulted in robust induction of NP-specific IgM and IgG1 in the serum of both groups, depicted in Figure 25. Rechallenge with soluble antigen but without adjuvant lead to rapid induction of memory responses, as expected, particularly for IgG1. However, again both groups responded similarly suggesting that memory formation in *Themis2*$^{KO/KO}$ mice is intact.

As alum is also used to prime animals for allergic reactions (see section 3.7.2) I also measured the production of total IgE in this system. Again I found no differences between both groups, either after primary or secondary immunisation.

I wondered whether the immunisation route might play a role so I immunised *Themis2*$^{KO/KO}$ mice and controls orally using cholera toxin. I measured cholera toxin-specific IgM, IgG1 and IgA antibody responses both in the serum and in the faeces but could not find any statistically significant differences (Figure 26A and B). In addition, I checked for germinal centre B cell and T follicular helper cell numbers in the Peyer's Patches, which were also equivalent between genotypes (Figure 26C). Furthermore, the number of Peyer's patches along the small intestine was

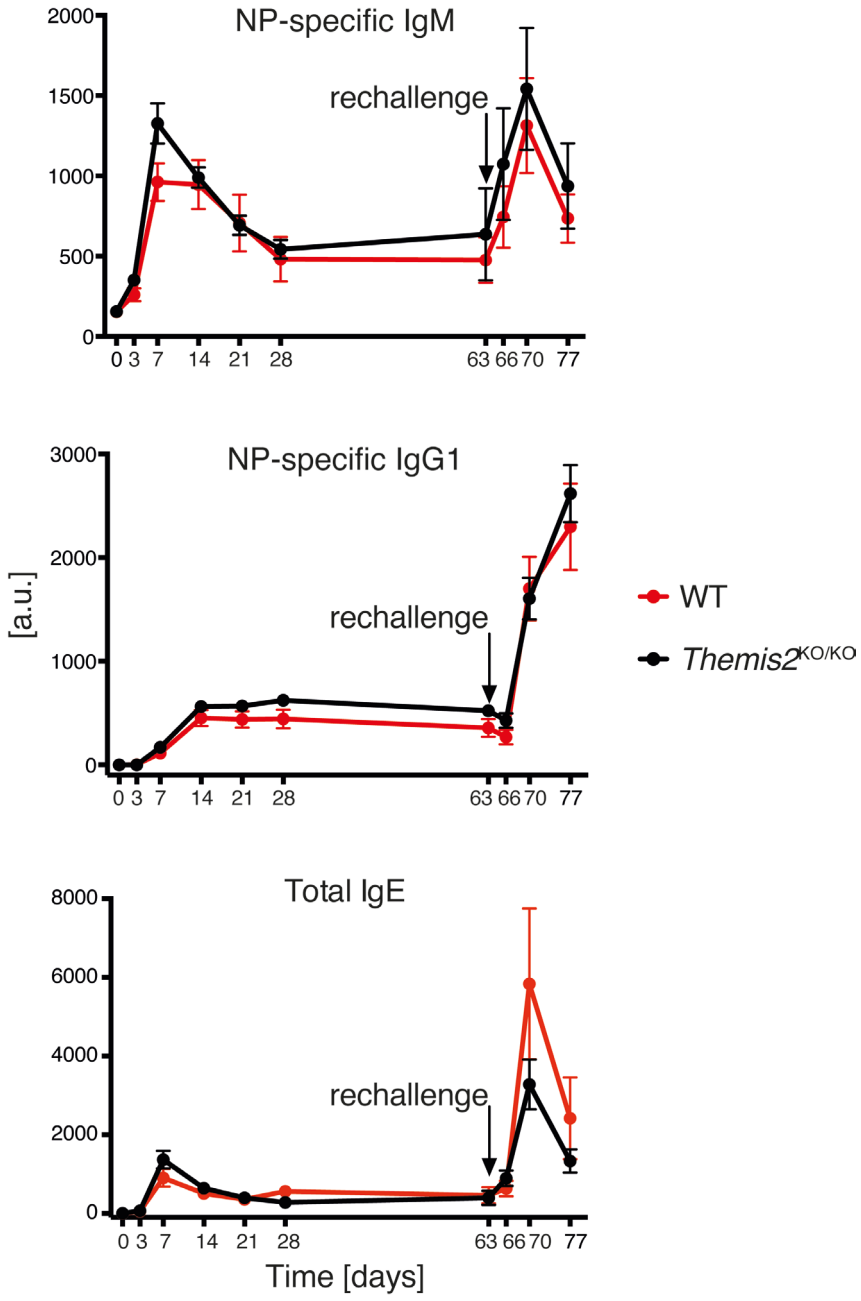

Figure 25: *Themis2*-deficient mice have normal primary and memory, humoral immune responses upon T-dependent immunisation with NP-CGG.

(A) Kinetics of NP-specific serum IgM and IgG1 or total IgE in WT and *Themis2*$^{KO/KO}$ mice following immunisation with NP-CGG in alum on day 0 to measure primary responses and rechallenge with NP-CGG in PBS on day 63 to test memory responses. Immunoglobulin levels were determined by ELISA. Graphs show mean ± SEM of 6 biological replicates/group. a.u., arbitrary units.

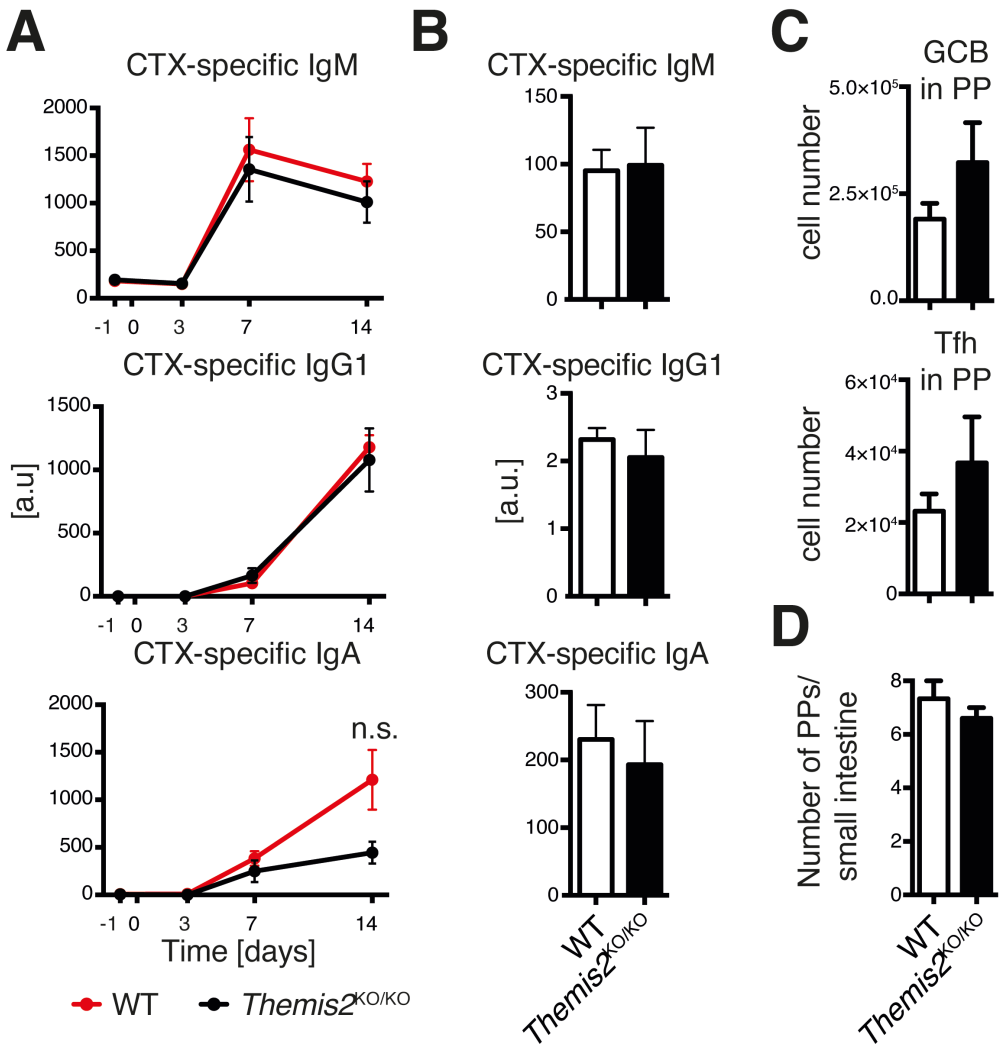

Figure 26: *Themis2*-deficient mice respond normally to oral immunisation with cholera toxin.

WT and *Themis2*$^{KO/KO}$ mice were orally immunised with cholera toxin (CTX) on day 0. **(A)** Kinetics of NP-specific serum IgM, IgG1 or IgA after CTX immunisation and **(B)** NP-specific faecal IgM, IgG1 or IgA on day 14 after CTX immunisation. **(C)** Cell numbers of germinal centre B cells (GCB, B220^{+}, CD38$^{low-int}$, GL7^{hi}, Fas^{hi}, PNA^{hi}) or T follicular helper cells (Tfh, TCRβ^{+}, CD4^{+}, CXCR5^{hi}, PD-1^{hi}) in the Peyer's patches (PP) and **(D)** number of Peyer's patches counted along the small intestine of CTX immunised mice on day 14. Immunoglobulin levels were determined by ELISA, cell numbers were determined by flow cytometry. Graphs show mean ± SEM of 5 – 6 biological replicates/group. a.u., arbitrary units; n.s., not significant.

also comparable between groups (Figure 26D). In summary, I found no indication that *Themis2*$^{KO/KO}$ mice have an impaired humoral immune response to T-dependent or T-independent antigens irrespective of the route of immunisation.

3.7 *Themis2*-deficiency in disease models

Finally, I sought to determine if *Themis2* has a role in actual disease models. I surmised that maybe in an even more complex immunological situation a phenotype for *Themis2* might be found as the exact immunological mechanisms that govern these responses are still a subject of intense research. It also gave the opportunity to test if any of the functions of other cell types, which express *Themis2*, are compromised in *Themis2*$^{KO/KO}$ mice.

3.7.1 Influenza infection

To test for a role of *Themis2* in a Th1 dominated immune response mice were infected intranasally with X31 influenza. Furthermore, this gave the opportunity to test for a function of *Themis2* in the lung as opposed to previous systemic (intraperitoneal) or gastrointestinal challenges. As this system is more complex and physiological compared to the previous immunisations, not only B cells play a role; also other *Themis2*-expressing cell types such as macrophages and dendritic cells could impinge on the outcome of an influenza infection through many different mechanisms

(Kreijtz et al., 2011). As *Themis2*^{KO} is a constitutive null allele these cell types too are deficient for *Themis2* and could also harbour defects.

Use of two different infectious doses ensured the ability to detect differences between groups as high doses lead to 83 % mortality rate and low dose only lead to 33 % mortality. However, I found no significant differences in animal weight loss (Figure 27A) or survival (Figure 27B) between *Themis2*$^{KO/KO}$ and wild type control groups. Production of IgG specific for X31 haemaglutinin tested 10 days after infection were also identical between genotypes (Figure 27C). All surviving mice also showed complete immunity to reinfection with X31 influenza at an 18.75-times higher dose than the high group confirming that immunological memory was intact (data not shown).

3.7.2 House dust mite allergy model

To study whether there was a function for *Themis2* in a Th2 cell-mediated disease I used a model of acute allergic lung inflammation. This also allowed me to test whether *Themis2* might play a role in eosinophils, as recently added publicly available data (www.immgen.org) indicate that *Themis2* is expressed in these cells as well. Therefore mice were primed with house dust mite in alum and then intratracheally challenged to induce acute airway inflammation. Slightly reduced infiltration was visible in histological sections stained with H&E or AB-PAS (Figure 28). I found the

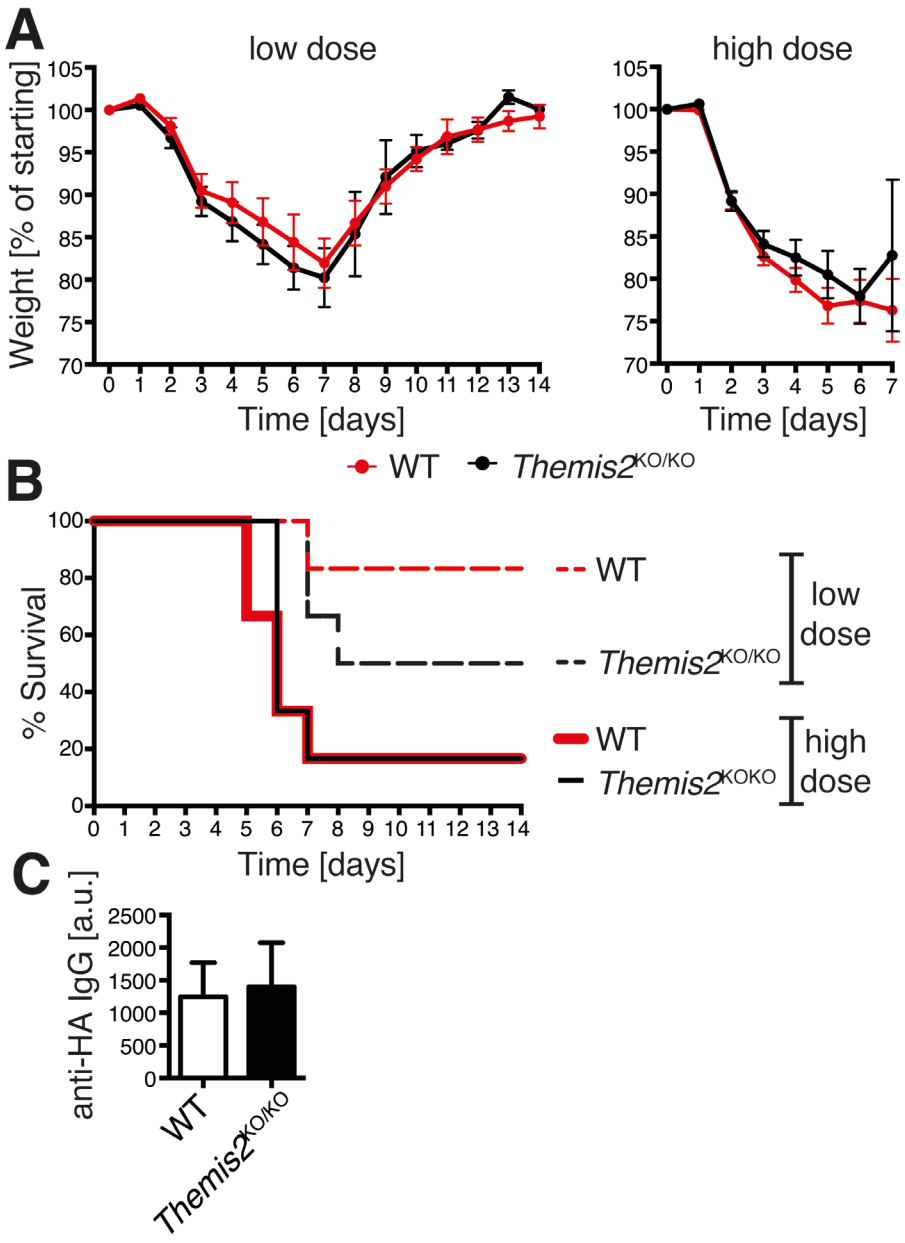

Figure 27: ***Themis2*$^{KO/KO}$ mice respond normally to influenza infection.**

WT or *Themis2*$^{KO/KO}$ mice were intranasally infected with X31 influenza $TCID_{50} = 8 \times 10^3$ (low dose) or 8×10^4 (high dose). **(A)** Weight loss of mice after infection on day 0. Due to high mortality, data beyond day 7 is not shown for the high dose group. Graphs show mean ± SEM of 6 mice/group. **(B)** Kaplan-Meier survival curve of WT or *Themis2*$^{KO/KO}$ mice from the experiment shown in (A). **(C)** Serum anti-HA IgG titre on day 10 after X31 influenza infection in the mice from experiment shown in (A). Bars indicate mean ± SEM of 4 – 6 mice/group; a.u., arbitrary units.

total cell count in bronchoalveolar lavages to be significantly reduced by half in *Themis2*-deficient mice (Figure 29A). Eosinophils, making up to 40 % of these infiltrating cells (Supplementary Figure 10A), were also reduced in absolute numbers (Figure 29B) although percentages of different cell types were not altered indicating that the overall cell infiltration was reduced. In contrast to the BAL cell counts, numbers of myeloid and lymphoid cells in the lung, as assessed by flow cytometry, were similar between genotypes (Figure 29C). Total serum IgE levels were also normal in *Themis2*$^{KO/KO}$ mice both before and after priming and after induction of acute airway inflammation (Figure 29D). I also analysed the expression of various goblet cell markers (*Gob5* and *Muc5ac*), alternatively activated macrophage markers (*Arg1*, *Fizz1*, *Ym1*) and the eosinophil chemoattractant *Eotaxin-1* in total lung RNA. The qRT-PCR results suggest that none of these are dysregulated (Figure 29E). Moreover, T cell signature cytokines (*IL-4*, *IL-5*, *IL-13*, *IL-17A* and *IFNγ*) in the lung were also not altered in the response (Supplementary Figure 10B). Overall these results suggest at best a very mild phenotype in the recruitment of cells into the bronchoalveolar lumen of the lung.

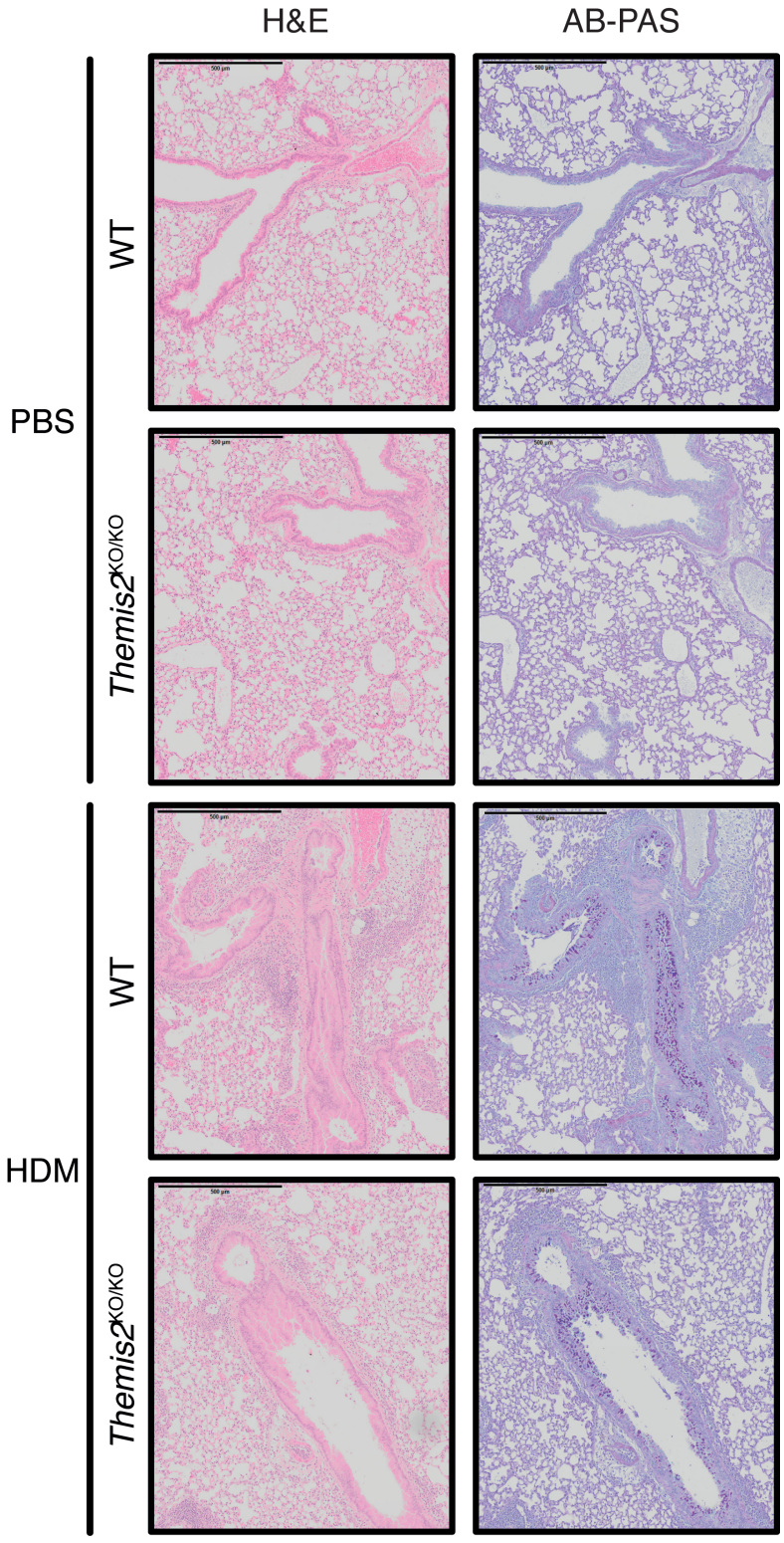

Figure 28: Mildly reduced infiltration into the bronchoalveolar lumen of *Themis2*$^{KO/KO}$ mice in a model of acute allergic inflammation to house dust mite.

Mice were sensitised to house dust mite (HDM) on day 0 and boosted on day 14 via intraperitoneal injection of HDM in alum. Then airways were

rechallenged intratracheally on day 28 and 31 with HDM in PBS before analysis on day 32. Controls received PBS without HDM. Histological sections of the left lung on day 32 stained with hematoxylin and eosin (H&E) or alcian blue periodic-acid schiff (AB-PAS) indicating cell infiltration (H&E) and mucopolysaccharide production (AB-PAS). Mildly reduced cell infiltration is visible in the HDM-treated *Themis2*$^{KO/KO}$ mice compared to HDM-treated wild type controls. Data representative of 2 independent experiments.

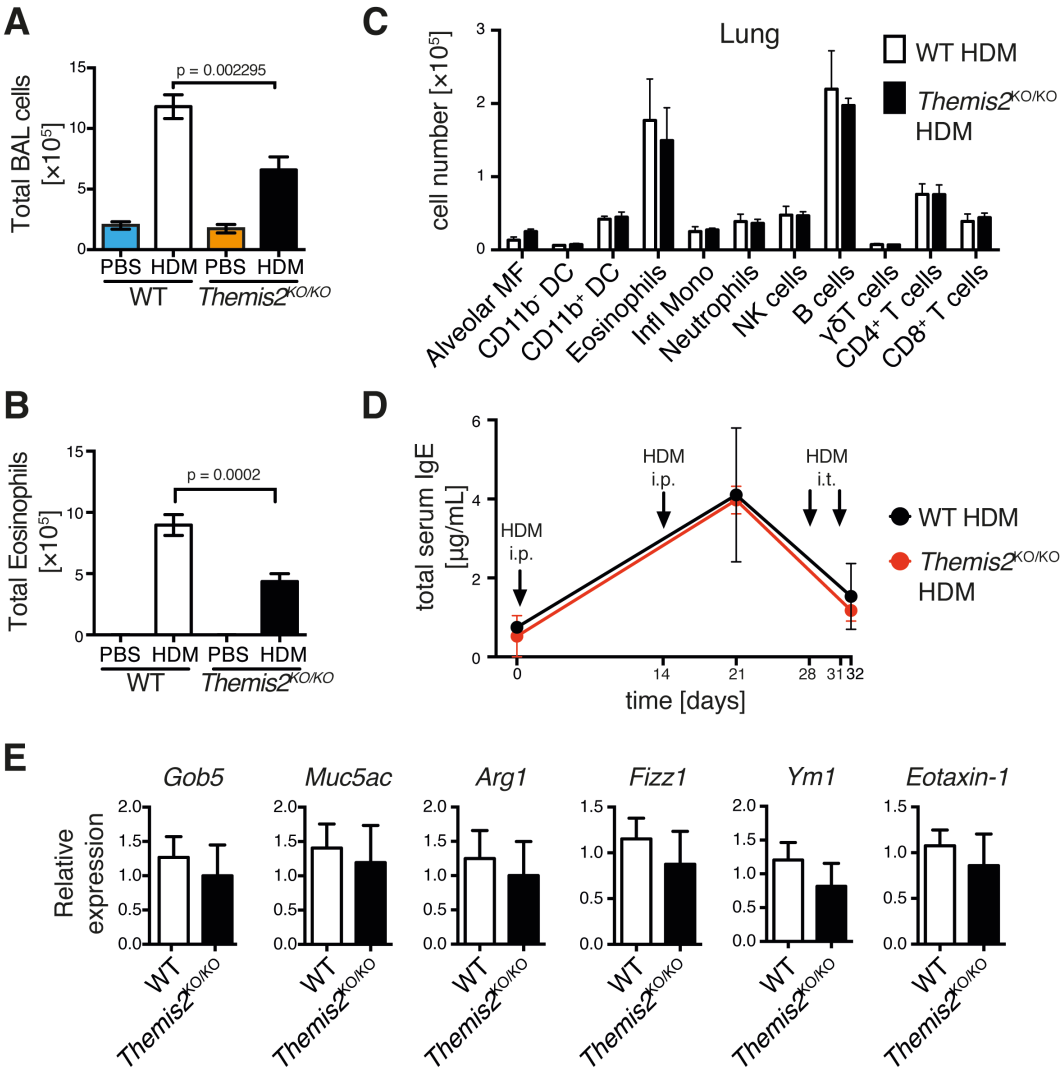

Figure 29: Reduced total cell numbers and eosinophils in the bronchoalveolar lavage fluid of *Themis2*-deficient mice in an acute house-dust mite allergy model.

Mice were sensitised to house dust mite as described in Figure 28. **(A)** Haemocytometric total cell count in the bronchoalveolar lavage fluid (BAL) of mice on day 32. Bars show mean ± SEM of two pooled, independent experiments with a total 3 (PBS) or 9 (HDM) mice/group. **(B)** Total eosinophil count in the BAL of mice on day 32 determined by cytospins. Bars show mean ± SEM of two pooled, independent experiments with a total 3 (PBS) or 9 (HDM) mice/group. **(C)** Total cell numbers of leukocytes in the inferior, middle and post-caval lobe of the lung of *Themis2*-deficient or control animals on day 32. Populations were analysed by flow cytometry and defined with the following markers: alveolar macrophages (Alveolar

MF, $CD45^+$ $SiglecF^+$ $CD11c^{hi}$ $F4/80^+$), $CD11b^-$ dendritic cells ($CD11b^-$ DC, $CD45^+$ $CD11c^+$ $F4/80^-$ $CD11b^-$), $CD11b^+$ dendritic cells ($CD11b^+$ DC, $CD45^+$ $CD11c^+$ $F4/80^-$ $CD11b^+$), eosinophils ($CD45^+$ $SiglecF^+$ $CD11c^-$), inflammatory monocytes (Infl Mono, $CD45^+$ $SiglecF^-$ $CD11c^-$ $CD11b^+$ $Ly6C^{hi}$), neutrophils ($CD45^+$ $CD11b^+$ $Ly6G^+$), natural killer cells (NK cells, $CD45^+$ $Ly6G^-$, $NK1.1^+$ $CD3^-$), B cells ($CD45^+$ $CD19^+$ $TCR\beta^-$), γδ T cells ($CD45^+$, $CD19^-$, $TCR\beta^-$, $TCR\gamma\delta^+$), $CD4^+$ T cells ($CD45^+$, $CD19^-$, $TCR\beta^+$ $CD4^+$ $CD8^-$) and $CD8^+$ T cells ($CD45^+$, $CD19^-$, $TCR\beta^+$ $CD4^-$ $CD8^+$). Bars show mean ± SEM of 4 – 5 biological replicates/group and are representative of 2 independent experiments. **(D)** Kinetics of total serum IgE determined by ELISA during the course of the model. Dots indicate mean ± SEM of 4 – 5 mice/group. **(E)** Relative mRNA expression in the superior lung lobe of *Gob5* and *Muc5ac* indicating goblet cell mucus production, *Arg1*, *Fizz1* and *Ym1* indicating alternative macrophage activation and *Eotaxin-1* (CCL11) for eosinophil recruitment. Bars show mean ± SEM of 4 – 5 biological replicates/group and are representative of 2 independent experiments. Statistical significance was assessed using the Mann-Whitney test.

4 Discussion

Data from previous studies suggested that *Themis2* might have a role in B cell biology. *Themis1*, the T cell-restricted, highly similar paralogue of *Themis2* has been shown to have a major role in the positive selection of thymocytes (Fu et al., 2009; Johnson et al., 2009; Kakugawa et al., 2009; Lesourne et al., 2009; Patrick et al., 2009). Since these two proteins showed mutually exclusive expression within T cells (*Themis1*) and B cells (*Themis2*) respectively I hypothesised that *Themis2* might have an equally important function in B cells compared to *Themis1* in T cells. Later, more evidence accumulated suggesting a role for *Themis2* in B cell development. The ENCODE project showed that typical B cell transcription factors such as PAX5, EBF1, PU.1 and NFκB bind in the promoter region of *Themis2* (Rosenbloom et al., 2013). Furthermore, data in macrophages suggested a role for *Themis2* in TLR4 signalling, a pathway which is found in murine B cells as well (Bekeredjian-Ding and Jego, 2009; Peirce et al., 2010). Lastly and most convincingly, it was shown that ectopic overexpression of *Themis2* in T cells can substitute for *Themis1* and rescue T cell development *in vivo* in the absence of *Themis1*. These results demonstrate that *Themis2* is a functional gene. Furthermore, this showed that THEMIS2 is rapidly phosphorylated after BCR cross-linking in primary B cells, again hinting at a function of *Themis2* in this cell type (Lesourne et al., 2012). An important role for *Themis2* is also suggested by its evolutionary conservation as two Themis family members can be

traced down to the sea lamprey (*Petromyzon marinus*) and are regularly found in ray-finned fish, *Xenopus tropicalis*, birds and mammals as evidenced by available sequences in ENSEMBL (Flicek et al., 2014; Johnson et al., 2009; Paten et al., 2008).

In this thesis I have addressed the question whether *Themis2* has a function in B cells. To this end, I first measured the expression of *Themis2* and other related Themis family members in developing, mature and antigen-experienced B cell subsets. Then, I used a newly created *Themis2*-deficient mouse strain to determine any defects in B cell development, activation, antibody responses and two disease models.

4.1 Expression of the Themis family

Combined results from PCRs and RNA sequencing as well as from FACS-Gal promoter studies indicated that *Themis2* is expressed throughout the B cell lineage with reduced expression in germinal centre B cells and recently activated or cultured B cells. Both *Themis1* and *Themis3* were not expressed in any of the analysed B cell subsets. In contrast, T cells expressed *Themis1* but did not express *Themis2*. *Themis3* was only detectable in the small intestine. Data for the other two CABIT domain-containing proteins *Garem* and *Gareml* also suggested that they are not expressed in murine B cells.

Although both qRT-PCR and FACS-Gal clearly show that *Themis2* is expressed in all B cell subsets there were slight differences in particular for pro-B and pre-B cells and transitional B cells. It should be kept in mind

that promoter activity and spliced transcript levels do not necessarily need to exactly correlate since for example rapid degradation of the transcript would lead to conflicting results between the promoter activity assay and qRT-PCR. Moreover, post-transcriptional processing of the mRNA could account for the subtle differences observed between these assays.

The FACS-Gal assay is also subject to the following caveats: First, brightness of the cells during flow cytometry depends on other parameters such as cell size which is presumably the reason why pro-B and pre-B cell results slightly differ between qRT-PCR and FACS-Gal. In the qRT-PCR pro-B cells gave lower levels compared to pre-B cells whereas it was the opposite in the FACS-Gal assay. Although the differences between pro-B and pre-B cells were not statistically significant they were nevertheless reproducible an all experiments. Pre-B cells have a bimodal size distribution in the forward scatter parameter, in accordance with large blasting pre-B cells becoming smaller pre-B cells during B cell development (Kurosaki et al., 2010). As assessed in the FACS-Gal, pre-B cells had more large cells compared to pro-B cells; pro-B had a lower number of large cells but cells were more spread out in the forward scatter parameter (data not shown). This could account for the small differences observed between those two subsets in the FACS-Gal assay and the qRT-PCR.

A second caveat is that the exclusion of dead cells is vital for a correct analysis of FACS-Gal assays since only intact cells can retain fluorescein. Cells with a normal forward and side scatter and staining profile (indicating

that the cell wall is mostly intact) would be assayed to have a non-active promoter due to tiny leaks in the plasma membrane leading to diffusion of the intracellularly produced fluorescein into the medium. Besides, it should be noted that the osmotic shock to load substrate into the cell leads to relatively high cell death even under optimised conditions, making a dead cell marker crucial. Different susceptibilities to osmotic shock among different B cell subsets could complicate the analysis further.

Thirdly, introduction of a reporter cassette could alter the locus leading to deregulation of the endogenous promoter, which in turn can lead to misleading results. The generally very good correlation between the FACS-Gal and qRT-PCR results suggests that the presence of the NeoFlox allele has only minor effects on the promoter activity, if it has any at all. However, any remaining minor differences could be accounted for by the alteration of the locus as FACS-Gal was measured in *Themis2*$^{+/NeoFlox}$ and qRT-PCR was measured in wild type mice respectively.

When B cells were put into culture, *Themis2* levels dropped, but even more so when cells were stimulated with anti-IgM, LPS or CD40L with IL-4 showing that *Themis2* expression is regulated upon B cell activation, hinting at a role for *Themis2* in B cell activation. mRNA levels of other CABIT domain containing proteins remained barely detectable and were not upregulated to potentially compensate for the downregulation of *Themis2*. In addition, no evidence was found for compensation by other CABIT-domain containing proteins due to upregulation of *Themis1*,

Themis3, *Garem* or *Garem1* in *a Themis2*-deficient background. In conclusion, the data indicate that *Themis2* is the only expressed CABIT-domain containing protein in murine B cells and there is no compensation by any of these related proteins. However, although unlikely, it is still possible that during the development of *Themis2*$^{KO/KO}$ B cells, upregulation of another CABIT-domain containing protein during some developmental stage masks a phenotype since expression of other CABIT-domain containing proteins during B cell development has only been assessed in wild type cells but not in *Themis2*$^{KO/KO}$ cells.

In terms of splice variants, *Themis2* was transcribed as a single messenger RNA containing all six exons as shown by PCR amplification of follicular B cell cDNA with amplicons stretching from exon 1 to exon 6 and by RNA sequencing of poly-A^{+} –enriched RNA libraries of follicular B cells. Since in B cells I was able to detect only one isoform for *Themis2,* all data so far indicate that the *Themis2* gene codes only for one protein product.

This is in contrast to reports in human cells where multiple splice variants have been identified (Treeck et al., 2002) although their physiological relevance so far is unclear. Interestingly, in this report differential splicing has not only been shown to occur between different exons but also within exon 4, which is also reported as isoform by other sources such as ENSEMBL and Uniprot with various degrees of supporting evidence. These splice sites follow the canonical GT-AG splicing rule and would delete a portion of the second CABIT domain.

Unless the assumption of the CABIT domain being a functional domain is wrong, none of the human splice variants seem to leave out particular domains but instead remove parts of one or multiple protein domains and would be predicted to rather obliterate the function of the whole protein. Several annotated human *Themis2* transcripts involve an excision of exon 4 (or excisions within exon 4). Exon 4 contains a phase change, so, as in mice, its deletion would push any downstream exons out of frame. Human *Themis2* exon 4 also contains the C-terminal end of the first CABIT domain, the whole second CABIT domain and the proline-rich SH3 binding site and therefore its deletion is very likely to produce a non-functional product. If however, the computationally predicted CABIT domain is not a domain *per se* then some of the reported splice variants could potentially lead to functionally different isoforms. It remains to be determined whether the reported transcript variants in humans actually produce distinct protein isoforms with distinct functional properties or whether some of the reported transcripts are degradation products or splicing intermediates. Structural studies would be needed to confirm or refute existence of the computationally-predicted CABIT domain.

4.2 Analysis of *Themis2*-deficient mice

To address the function of *Themis2*, I created *Themis2*-deficient *Themis2*$^{KO/KO}$ mice. qRT-PCR and RNA sequencing confirmed the deletion of *Themis2* exon 4 in *Themis2*$^{KO/KO}$ mice and immunoblots corroborated the ablation of full-length THEMIS2 protein (molecular weight = 74.4 kDa)

and supported the prediction that any truncated THEMIS2 had a frame shifted C-terminus since an antibody to an epitope in exon 6 did not detect any smaller proteins. The truncated form of THEMIS2 would be expected to have a molecular weight of 24.2 kDa (exons 1, 2, 3) or 32.3 kDa (frame shifted product of exon 1, 2, 3, 5, 6) if exon 6 were still translated in frame. However, this was additionally ruled out by RNA sequencing. Faint remaining bands in some of the immunoblots appear to be cross-reactions with proteins of similar molecular weight because they were not consistent in between all anti-THEMIS2 antibodies. Additionally, genotyping PCRs of gDNA and qRT-PCRs of *Themis2* transcripts showed that *Themis2* exon 4 is not detectable in *Themis2*$^{KO/KO}$ B cells and full-length THEMIS2 protein can therefore not be produced anymore.

4.2.1 Possible limitations to the analysis of *Themis2*$^{KO/KO}$ mice

Since, unfortunately, none of the antibodies to the N-terminal region of THEMIS2 that I tested were able to detect THEMIS2, I was unable to determine whether a truncated and C-terminally frameshifted mutant of THEMIS2 was produced in *Themis2*$^{KO/KO}$ B cells. That a truncated mutant of THEMIS2 is still produced in these mice is a possibility as qRT-PCRs indicate that the mutant *Themis2* transcript is produced at normal levels at the exon junctions 2 – 3 and 5 – 6 and splicing was found from exon 3 to exon 5. RNA sequencing data verified the qRT-PCR data and confirmed the expected frameshift. Normal expression of the truncated transcript is expected as it would be predicted that nonsense-mediated RNA decay

would fail to recognise the truncated transcript as aberrant. In the *Themis2*^{KO} allele the first frameshifted, premature termination codon (PTC) appears in the last exon of the gene, exon 6, just less than 50 bases from the original stop codon. As a consequence, the nonsense-mediated RNA decay machinery would not be predicted to recognise the transcript as aberrant since there are neither exon junction complexes downstream of the first PTC nor does the length of the 3' UTR change significantly (Schweingruber et al., 2013).

If the N-terminal fragment of THEMIS2 would be translated and stable it would be comprised of the first 214 amino acids of CABIT1 lacking the last 23 C-terminal amino acids of the domain. The frameshifted exons 5 and 6 would give rise to a 74 amino acid-long tail after the truncated CABIT1 domain, giving rise to a protein of 288 residues in total compared to 663 amino acids for full-length THEMIS2 (Supplementary Figure 5). However, it is unlikely that such a fragment would sustain the function of *Themis2* for several reasons:

Firstly, this fragment lacks both the C-terminal, SH2-binding, phosphorylation site Y660 and the conserved PRS. Both for *Themis2* and *Themis1* it has been shown that the PRS is required for GRB2-binding (Paster et al., 2013; Peirce et al., 2010).

Secondly, GRB2-binding has been shown to mediate THEMIS1 recruitment to LAT and that this recruitment is essential for thymocyte development (Paster et al., 2013). Likewise, when *Themis2* was ectopically expressed in T cells, it was shown to be recruited to LAT and it

functionally substituted THEMIS1, restoring thymocyte development (Lesourne et al., 2012). These results suggest that both *Themis1* and *Themis2* function by the same mechanism, namely PRS-site mediated recruitment via GRB2 to the signallosome. The truncated fragment could consequently not perform this function as it lacks the PRS.

Thirdly, both the *Themis2* PRS and Y660 have been shown to be critical for modulation of TNFα production in macrophages and Y660 has been essential for THEMIS2 phosphorylation and LYN recruitment (Peirce et al., 2010), all of which would be abrogated in the absence of these residues in the remaining fragment.

Fourthly, a point mutation in the C-terminal end of the second CABIT domain of *Themis1* leading to a premature stop codon (Y489X) also abrogated thymocyte development *in vivo*. This mutation affects a similar but slightly smaller part of the protein compared to the *Themis2*^{KO} allele and again argues that the fragment of THEMIS2 that may potentially remain in *Themis2*$^{KO/KO}$ mice is not functional.

Lastly, it has been shown that expression of *Themis1* mutants of the core of CABIT1 or the core of CABIT2 or the PRS or the NLS fail to restore the function of *Themis1* in *Themis1*-deficient mice, suggesting that all of these functional domains are essential for the function of Themis proteins. Of interest though are results showing that if only the conserved cysteines of both CABIT domains were mutated these mutants restored thymocyte development almost to wild type levels (Zvezdova et al., 2014). It has previously been speculated that these cysteines might serve a catalytic

function (Johnson et al., 2009). These results indicate that these two amino acids are of minor importance whether they serve a catalytic function or simply a structural function. If they were serving a catalytic function, it is dispensable for T cell development (Okada et al., 2014; Zvezdova et al., 2014). Overall, these data strongly suggest that the truncated THEMIS2 protein would not be able to sustain its function.

It is difficult to assess whether the frameshifted C-terminal tail in the truncated THEMIS2 mutant could influence protein stability. The expression of the truncated THEMIS2 protein in *Themis2*$^{KO/KO}$ animals might be possible as it is a transcript with a 3' UTR and polyadenylation. On the one hand the frameshifted C-terminal tail could destabilise the protein and lead to its degradation, on the other hand, although less likely, it could help stabilise truncated THEMIS2 and help maintain THEMIS2 functions by higher protein levels of mutant THEMIS2 or not change the properties of the protein turnover at all.

In the course of generating Themis2$^{KO/KO}$ mice, I additionally analysed mice homozygous for the intermediate allele *Themis2*^{NeoKO} (*Themis2*$^{tm1b(KOMP)Wtsi}$) in which *Themis2* exon 4 is deleted but the reporter cassette is still in place. Preliminary data indicated that the presence of the reporter cassette with a splice acceptor site and a polyadenylation site lead to almost completely abolished transcription of exon 5 and 6 indicating that even if the truncated protein was produced it would mostly consist of exon 1, 2 and 3. Yet, I did not find any developmental defects in these mice either (data not shown). This further supports the validity of the

Themis2$^{KO/KO}$ results as also in the absence of the frameshifted novel C-terminus, truncated *Themis2* is dispensable for B cell development.

With the caveat in mind that following deletion of *Themis2* exon 4 a truncated THEMIS2 could still be expressed in Themis2$^{KO/KO}$ mice, I argue that exon 4 is the best exon to target in *Themis2* for creation of both constitutive and conditional knockout mice since it introduces a frameshift in downstream exons and affects all functionally annotated domains and sites. All other exons are smaller than exon 4 and deletion of exon 1 could lead to reinitiation of translation, potentially yielding only a short N-terminal truncation of THEMIS2. In addition, targeting exon 1 could interfere with the promoter and normal transcription and is thus unsuitable for a conditional mutant and/or reporter constructs. In contrast, deletion of exons 2 or 3 would only excise part of CABIT1 and, since they are phase symmetric, leave downstream exons in frame; deletion of exon 5 or 6 would only delete part of the C-terminus and leave CABIT1, CABIT2 and the PRS intact. Targeting the whole gene or multiple exons for deletion could result in ineffective conditional deletion due to the size of the gene and the distance between the loxP sites. Therefore, targeting exon 4, as in the *Themis2*$^{KO/KO}$ mice, produces the most comprehensive null mutation of *Themis2* if a conditional allele is desired too, which is a useful tool to analyse any *Themis2* phenotypes in more detail.

4.2.2 B Cell development

I found B cell development in Themis2$^{KO/KO}$ mice to be normal since all B cell subsets were detected in equal numbers and cell surface markers did not change either. Even under competition with wild type cells, *Themis2*-deficient B cells developed normally as demonstrated by the mixed bone marrow radiation chimera experiments. This was surprising as *Themis1*-deficiency leads to reduced single positive thymocytes and peripheral T cells due to a defect in selection during T cell development (Fu et al., 2009; Johnson et al., 2009; Kakugawa et al., 2009; Lesourne et al., 2009; Patrick et al., 2009). However, T cell selection and B cell selection differ in some aspects as discussed below in section 4.2.6, which might give reasons for the difference observed between the two phenotypes.

Indistinguishable usage of variable region genes suggests that the B cell repertoire is comparable as well, although there may be differences in combinations of VDJ genes used. If *Themis2*-deficiency would lower the negative selection threshold for BCR signalling similar to *Themis1*-deficiency in for TCR signalling (Fu et al., 2013) then one would expect to find either more deletion and anergic cells, which was not the case as shown by cell numbers, or at least a changed repertoire due to receptor editing.

Identical usage of in particular light chain variable regions and of κ and λ constant regions also argues against changes in B cell selection due to increased receptor editing since receptor editing often leads to changes in light chain recombination, first rearranging at the κ locus and if

unsuccessful at the λ locus (Arakawa et al., 1996; Klein et al., 2004; Nemazee, 2006).

Whole transcriptome analysis of follicular B cells showed no substantial differences in gene expression. The few, identified differentially expressed genes did not give a clear picture of any affected pathways. Cellular pathways and functions in which the number of differentially regulated genes was statistically overrepresented and which thus might have been affected such as “cellular development” or “cell activation” have been tested but did not reveal any differences.

4.2.3 B Cell activation

Themis2 is the sole CABIT domain containing protein expressed in follicular B cells at steady state and after stimulation with anti-IgM, LPS, or CD40L with IL-4. When put into culture *Themis2* expression drops but even more so if cells are stimulated with the above stimuli. The immediate downregulation of *Themis2* suggests a function in cell activation and is recapitulated in splenocytes after influenza infection and in lymphocytes and macrophages after activation in a collagen-induced arthritis model (Peirce et al., 2010).

Nevertheless, stimulation through either the BCR, TLR4, CD40, IL-4R or BAFFR in *Themis2*-deficient B cells results in equal expression of surface activation markers CD23, CD69, CD86 and MHC class II. Moreover, expression of 16 different cytokines as well as proliferation and survival are indistinguishable from the wild type. Normal production of IL-10 by

Themis2-deficient B cells implied that B regulatory cell development and function are most likely also unaffected by the absence of *Themis2*.

Gene expression profiles of follicular B cells after activation failed to reveal clear defects as well. However, under several conditions I found a few significantly differentially regulated genes involved in cholesterol metabolism. It is interesting to note that also *Themis1*-deficient thymocytes have been reported to have dysregulated genes involved in cholesterol metabolism but the significance of this finding has remained elusive so far (Johnson et al., 2009). In B cells, availability of cholesterol has been implicated in BCR signalling (Karnell et al., 2005) and BCR internalisation (Bléry et al., 2006). However, the significance of these differentially regulated cholesterol metabolism genes remains obscure since BCR internalisation was completely identical between *Themis2*-deficient and control B cells and no defects in BCR signalling were observed either. It is possible that very minor signalling defects might be detectable by biochemical methods but in the light of not having any downstream consequences I decided not to pursue immediate BCR signalling events any further.

Instead, to test whether further downstream interactions with T cells were defective, I performed an antigen presentation assay by targeting OVA antigen to the cross-linked BCR and co-culturing the antigen-loaded B cells with OT-II T cells. T cell activation was read out by measuring proliferation via CTV dilution and IL-2 secretion in the supernatant. In the optimised assay, both parameters allowed detection of concentration-

dependent T cell activation in response to different amounts of BCR cross-linking and antigen loading. Different antigen loading was also confirmed on the B cell surface by flow cytometry (Supplementary Figure 9). Although B cells were not purified by flow cytometry for this assay, the antigen presentation was not performed by contaminating myeloid APCs as when B cell isolations were incubated without anti-IgM to target the antigen to the BCR but with high amounts of OVA antigen as shown in Figure 21B “Antigen-loading: none”, T cells were not activated, indicating that pinocytosis of antigen and subsequent antigen presentation by contaminating APCs other than B cells was insufficient to cause T cell activation. Furthermore, antigen presentation was an active process, as fixed B cells could not activate T cells either. Cultures also needed both B and T cells to produce detectable levels of IL-2 and T cell proliferation. The results show that *Themis2* is dispensable for antigen presentation to $CD4^+$ T cells as both proliferation and IL-2 production in the assay were identical between *Themis2*-deficient B cells and wild type controls.

In summary, early events after B cell activation are normal in B cells from $Themis2^{KO/KO}$ mice. B cell activation was normal as assessed by surface markers, survival, proliferation, cytokine production, BCR internalisation, antigen presentation and transcriptome analysis after activation by four different stimuli.

4.2.4 B Cell antibody responses

In order to test for more long-term effects of *Themis2* after B cell activation I measured antibody responses to various types of antigens in *Themis2*$^{KO/KO}$ mice. To test whether mice started off at equivalent antibody levels before immunisation, I first measured total antibody levels in unimmunised mice and found that all seven mouse isotypes were present at equal levels in the serum of *Themis2*$^{KO/KO}$ and wild type control mice.

Next, I tested a type I T-independent antigen NP-LPS, which, in addition to BCR ligation, conveys a second signal. In the case of LPS, receptors such as TLR4 are activated. I was particularly interested in NP-LPS since it has been shown for LPS-stimulated macrophages that if *Themis2* is overexpressed by transfection or silenced using RNAi, TNFα production is increased or reduced respectively. This was attributed to regulation of p38 and ERK, the phosphorylation of which was increased after LPS stimulation if *Themis2* was overexpressed (Peirce et al., 2010).

Thus, I immunised mice intraperitoneally with NP-LPS in alum and followed the NP-specific antibody response in the serum of the mice for four weeks. However, control and *Themis2*$^{KO/KO}$ mice responded equally well to the immunisation with all analysed parameters including NP-specific IgM, IgG1, IgG2b, IgG2c and IgG3. Production of splenic germinal centre B cells, plasmablasts and plasma cells was also normal in *Themis2*-deficient mice as assessed on day 5 of the response.

Since this study was focused on B cells, I did not measure serum TNFα production as B cells produce much less TNFα than macrophages and

most of the serum TNFα would be attributed to production by myeloid cells. However, it is interesting to speculate whether TNF production in *Themis2*$^{KO/KO}$ mice is deregulated. It is unlikely that altered TNFα levels, potentially caused by *Themis2*-deficiency, could cause a change in the antibody response to NP-LPS. *Tnf*-deficient mice can form antibody responses but seem to have defects generating germinal centres. Immunisation with the type 1 T-independent antigen Trinitrophenyl (TNP)-LPS in *Tnf*-deficient mice results in normal anti-TNP IgM production and an only marginally reduced anti-TNP IgG responses (Pasparakis et al., 1996). Thus it seems unlikely that a smaller reduction in TNFα production, as reported *in vitro* for human *Themis2*-silenced, LPS-stimulated primary macrophages (Peirce et al., 2010), could substantially influence the NP-LPS responses in *Themis2*$^{KO/KO}$ mice.

Moreover, preliminary experiments with bone marrow derived macrophages (BMDMs) from *Themis2*$^{KO/KO}$ mice or wild type littermate controls did not support the siRNA and overexpression studies by Peirce et al. which showed that RAW macrophages overexpressing *Themis2* produce more TNFα and human primary macrophages transfected with two different small interfering RNAs (siRNAs) against *Themis2* produced less TNFα in response to LPS. I found that *Themis2*$^{KO/KO}$ BMDMs produced equal amounts of TNFα after LPS stimulation compared to wild type controls (data not shown). Similarly to BMDMs, also B cells responded with identical TNFα production to LPS (Figure 19) and showed equal antibody responses to other immunisations and equal resistance to

influenza infection and allergy (Figures 24 to 29). One would expect that if TNFα production were severely reduced in *Themis2*$^{KO/KO}$ mice, these responses would be affected. For example TNFα has complex roles during influenza infection depending on the cell type which produces it and reduced TNFα generally improves survival during influenza infections (DeBerge et al., 2014; Hussell et al., 2001; Peper and Van Campen, 1995) yet Themis2$^{KO/KO}$ mice responded equally well to influenza infection suggesting that TNFα production is most likely not strongly deregulated in *Themis2*$^{KO/KO}$ mice.

A possibility to explain the observed discrepancies in LPS-induced TNFα production by macrophages reported by Peirce *et al*. and the preliminary BMDM data generated here could be that there are differences between the primary, murine BMDMs used here and the murine RAW macrophage cell line and the primary human macrophages used in the study by Peirce et al. It is possible that the findings represent an artefact found in the murine RAW cell line, which is not found in primary, murine BMDMs but fortuitously an actual phenotype in human primary macrophages. Alternatively, although unlikely, both siRNAs that were used in the study might have had off-target effects both leading to reduced TNFα production and thus leading to the conflicting results. Controls in this siRNA experiment used a pool of non-targeting siRNAs and not just transfection reagent, thus changes due to an interferon response to the siRNA by the macrophages were excluded.

In any case, *Themis2*-deficiency after injection of NP-LPS did not appear to impinge on B cell function in terms of cell differentiation or antibody production, irrespective of whether this was connected to a change in TNFα production or not.

Next, I tested a type II T-independent antigen, NP-Ficoll, in a similar manner to NP-LPS. Measurement of NP-specific IgM and IgG3 in the serum of *Themis2*$^{KO/KO}$ mice or controls did not show any differences suggesting that the response to repetitive non-protein antigens was also intact.

To model the last type of antigen immunisation I used NP-CGG in alum. This T-dependent immunisation induced a rapid NP-specific IgM response followed by increases in NP-specific IgG1 and total IgE in both experimental groups. Reimmunisation with soluble antigen induced a strong IgG1 and IgE memory response and, as expected, a more rapid IgM response. However both groups responded equally well suggesting that not only primary antibody responses but also memory responses were intact in *Themis2*$^{KO/KO}$ mice.

As the route of immunisation might influence the outcome, I also tested an oral T-dependent immunisation with cholera toxin. This would target immune cells in the gut more than previous intraperitoneal immunisations and might reveal differences. However, I found cholera-toxin specific IgM, IgG1 and IgA in both serum and faeces to be comparable between experimental groups. In addition, numbers of germinal centre B cells and T

follicular helper cells as well as the number of Peyer's patches was unchanged in *Themis2*$^{KO/KO}$ mice.

In summary, switching to each mouse isotype was checked in least one immunisation but no differences were detected. Overall the results conclusively suggest that *Themis2* is dispensable for normal antibody responses. Although not directly tested in more detail, it also suggests that macrophages and dendritic cells, which have also been reported to express *Themis2*, function largely normal in the absence of *Themis2 in vivo* as functions such as antigen presentation are required for a B cell response as well.

4.2.5 Infection and allergy models

Finally, to evaluate the contribution of *Themis2* to resistance to disease and reveal any defects in particularly complex immunological reactions in which cell types other than B cells also play major roles, I challenged *Themis2*-deficient mice with an influenza infection and a model of acute allergic lung inflammation.

Influenza infection was chosen as it is a mainly Th 1 cell driven viral infection (Brown et al., 2004) but also Th17 cells and Tregs have been found to affect the course of the disease (Kreijtz et al., 2011). Influenza infection caused equal weight loss and mortality at two different doses in controls and *Themis2*$^{KO/KO}$ mice and anti-haemagglutinin IgG was also produced normally (Figure 27). In addition, surviving mice were reimmunised and showed complete immunity to influenza infection and

increased antibody titres (data not shown). In conclusion, the data reveal that *Themis2* is not required for resistance to influenza infection.

To also test *Themis2*-deficient mice in a Th2-dominated disease I used a model of acute allergic lung inflammation using house dust mite as the model allergen. I found that IgE levels, goblet cell markers, alternatively activated macrophage markers, cytokines and chemokines, and cell recruitment in the lung were comparable between genotypes. However, I found a small, statistically significant difference in the total cell count of the bronchoalveolar lavage fluid. *Themis2*$^{KO/KO}$ mice had reduced BAL cell counts, which was concurrent with a reduction in the number of eosinophils in the BAL. Recently added, publicly available data (www.immgen.com) indicate that *Themis2* is also expressed in eosinophils. I investigated whether the reduced eosinophilia was due to a reduction in the eosinophil chemoattractants *Eotaxin-1* or *IL-5* (Conroy and Williams, 2001) but both of them were expressed at normal levels in lung tissues as assessed by qRT-PCR. Analysis of BAL cells by flow cytometry surprisingly revealed that the percentage of eosinophils in the BAL compared to other cell types was unchanged suggesting that recruitment into the BAL in general was impaired and that the eosinophil count was statistically significant due to these cells representing the major infiltrating cell type (~ 40 % of live cells, Supplementary Figure 10). Considering the number of individual tests performed on *Themis2*$^{KO/KO}$ mice, multiple testing becomes a statistical problem. The reduction of only approximately 44 % with a p-value of 0.002295 argues that the sample size should be

increased (currently n = 10 for wild type and n = 9 for *Themis2*$^{KO/KO}$) to confirm the reduction in total BAL cells and eosinophils (and possibly other cell types) as they stand in contrast to a lot of negative data even in the same experiments. It was surprising that all analysed cell types and not for example eosinophils in particular are recruited in lower numbers into the bronchoalveolar lumen. This could suggest that the defect might be rather found in the epithelium than in immune cells. However, *Themis2* expression is very low both in unstimulated and influenza-infected murine, primary airway epithelium cultures from the trachea (Andreas Wack, personal communication) and would suggest an indirect mechanism possibly influenced by a *Themis2*-expressing immune cell.

In summary, apart from the slight reduction in BAL cells, *Themis2*$^{KO/KO}$ mice responded indistinguishably to induction of acute allergic airway inflammation. It still remains to be determined whether the reduced BAL cell count can be consistently observed or whether it was due to experimental variation.

4.2.6 Possible explanations for the failure to detect a developmental phenotype due to *Themis2* deficiency

In the study presented here the function of *Themis2* in B cells was analysed, however no clear defects in *Themis2*-deficient mice, were observed so far. The lack of a phenotype could be explained by the *Themis2* gene not being functional. This is unlikely for two reasons. First, *Themis2* is found in all vertebrates. This evolutionary conservation

indicates that *Themis2* is likely to have an important function (Johnson et al., 2009). The second possibility is that the *Themis2* gene in laboratory mice has become non-functional. However, this is also improbable because mouse *Themis2* rescued *Themis1*-deficiency if ectopically expressed in the T cell lineage thus restoring T cell development to normal levels (Lesourne et al., 2012). Overall, these findings support that *Themis2* is functional, yet its specific function remains obscure.

Interestingly, *Themis1*-deficiency has a dramatic effect on developing T cells while *Themis2* seems dispensable for B cell development. The developmental results presented here thus support the view that selection of B and T lymphocytes during development is different to a certain degree. There are several indications that the selection process is much more stringent for T cells than for B cells, which could help explain why *Themis1* has an important role in lymphocyte selection and why *Themis2* does not.

For example, stringency in T cell selection is much more important because failure of self tolerance in the T cell compartment would also enhance autoreactive B cell responses (Xing and Hogquist, 2012). Conversely, B cells have been shown to have a relatively high number of autoreactive cells both in mice and men, being around 75 % of all clones in early immature B cell clones and around 40 % of newly emigrated mature B cell in human blood (Grandien et al., 1994; Wardemann et al., 2003).

The importance of T cell selection is also shown by the fact that T cells have the thymus as a dedicated organ for this process whereas niches for positive and negative selection of B cells in the bone marrow are not completely clear (Pelanda and Torres, 2012), but a niche of stromal cells expressing CXCL12, IL-7 and Galectin-1, with which developing B cells sequentially interact to support B cell development, have been reported (Mourcin et al., 2011; Tokoyoda et al., 2004). Although there is clear evidence for negative selection in B cells, apoptosis as an outcome is only a fail-safe mechanism in B cells to prevent autoreactive clones from reaching maturity if other mechanism such as receptor editing, anergy and ignorance fail. Compared to T cells in which about 75 % of thymocytes subjected to selection are deleted in the process (Sinclair et al., 2013), clonal deletion in B cells is less frequent. The extent of deletion in B cells is currently unknown but might be insignificant based on repertoire and receptor editing studies showing 50 – 70 % of immature B cells are autoreactive and 30 – 35 % edit their receptors. The remaining 15 – 40 % must therefore undergo either anergy, ignorance or clonal deletion, thus showing that B cell selection is more lenient (Grandien et al., 1994; Pelanda and Torres, 2012; Wardemann et al., 2003).

Themis1 appears to be regulating the signalling threshold for positive and negative selection in T cells but such a threshold for negative selection in B cells seems higher, indicated by for example the higher autoreactive B cell pool and alternative mechanisms of avoiding self-reactivity such as receptor editing. Lack of *Themis1* leads to a lowering of the signalling

threshold for negative selection, so thymocytes that would normally be positively selected due to a low to intermediate-strength TCR signal are negatively selected in the absence of *Themis1*. The usually positive-selecting signal is interpreted as a high affinity self-ligand signal leading to activation-induced apoptosis (Fu et al., 2013). Whereas *Themis1* is crucially involved in the selection process, I did not find any evidence for a similar function of *Themis2* in B cells. Potentially, *Themis2* also regulates this selection threshold in B cells but its effects might not be as dramatic as the ones of *Themis1* in T cells or the threshold can be moved more in B cells without strongly affecting the outcome of B cell selection. It is possible however that the methods used here including B cell development in intact and mixed bone marrow chimeras and analysis of VDJ gene and Igκ and Igλ usage, are too crude to detect small differences in selection. Perhaps more rigorous analyses on a monoclonal BCR transgenic background could have identified such a function.

In summary, the teleologically different requirements for positive selection between B and T cells, the higher negative selection thresholds in B cells evidenced by more autoreactive clones in the repertoire combined with the mechanism by which *Themis1* affects T cell selection i.e. dampening of TCR signals to fall into the positive selection range, might offer some indications for the lack of phenotype in B cell development of *Themis2*-deficient mice compared to the relatively strong defect in T cell development in *Themis1*-deficient mice. If *Themis2* performs a similar function in B cells to *Themis1* in T cells then perhaps the threshold for B

cell negative selection is not lowered enough in the absence of *Themis2* to see any functional consequences in a polyclonal repertoire.

In view of this, one can speculate that the different requirements for *Themis1* and *Themis2* might be connected to the different requirements for *Lat* and *Lat2* in T cells and B cells respectively. Similarly to THEMIS2, LAT2 is not required for B cell development (Fuller et al., 2011). In contrast, the presence of LAT is crucial for T cell development and so is the recruitment of THEMIS1 to LAT (Paster et al., 2013; Zhang et al., 1999) whereas *Lat2*-deficient mice show normal development of B cells (Wang et al., 2005; Zhu et al., 2004). Evidence suggests that LAT is important in T cells for the recruitment of SLP-76 to the plasma membrane whereas LAT2 is dispensable for the recruitment of the SLP-76 paralogue BLNK (Boerth et al., 2000; Engels et al., 2001; Ishiai et al., 2000; Zhang et al., 1998). It is interesting to speculate that maybe THEMIS1 is involved in the recruitment of SLP-76 to LAT. Assuming that the signalling mechanism of THEMIS2 is identical to the one of THEMIS1 and since LAT2 has been proposed to be a functional equivalent to LAT without a PLCγ-binding site (Fuller et al., 2011), it is possible that THEMIS2 may be recruited to LAT2 but since LAT2 is not required for BLNK plasma membrane localisation in B cell development, THEMIS2 is not required either because the signalling mechanism for the recruitment of BLNK in B cells is different to the one for SLP-76 in T cells. However, the molecular mechanism of THEMIS1 is potentially very different because it has been reported that THEMIS1 recruits SH2-domain-containing protein tyrosine phosphatase 1 (SHP-1),

suggesting a different role signalling. Moreover, SLP-76 phosphorylation is unaffected in *Themis1*-deficient thymocytes after TCR engagement (Fu et al., 2013) and THEMIS1 phosphorylation is dependent on SLP-76 (Brockmeyer et al., 2011) again suggesting that Themis1 acts downstream of SLP-76. Similarly LAT2 could have completely different functions to THEMIS2 and not associate with it at all. Whatever the exact mechanism, it is interesting to note the parallels between *Themis2* and *Lat2* with respect to the known interactions of THEMIS1 and LAT.

Furthermore, although not expressed in mature B cells, *Lat* has been shown to have a role during pre-BCR signalling and *Lat*-deficient mice show a minor accumulation of $B220^{+}$ $CD43^{+}$ B cells (Su and Jumaa, 2003). THEMIS2 has been shown to be able to bind to LAT (Lesourne et al., 2012) and could therefore be required for LAT signalling in early B cell development but again I did not observe any accumulation at the pro B cell stage. This indicates that LAT in early B cells signals via pathways that do not depend on THEMIS2.

4.3 Future directions

In conclusion, the results presented here exclude a role for *Themis2* in most major B cell functions notwithstanding that less recognised processes in which B cells are involved could still critically depend on *Themis2*. For example, I have not explored the requirement for *Themis2* in autoimmunity, which might be particularly interesting if there is a role for *Themis2* in B cell selection. Furthermore, I have not tested the role of

Themis2 in helminth, fungal, bacterial or mycobacterial infections. For bacterial infections there is recent, limited evidence from the phenotyping effort of the Mouse Genetics Project at the Wellcome Trust Sanger Institute (WTSI). They report that *Themis2*$^{NeoFlox/NeoFlox}$ mice respond normally to *Salmonella typhimurium* and *Citrobacter rodentium* challenge (data publicly available through their mouse resources portal, www.sanger.ac.uk/mouseportal). We know from our studies that this allele is hypomorphic, where *Themis2* expression is strongly reduced but most likely not completely absent (data not shown), leaving the possibility that low levels of *Themis2* could still sustain its function. Moreover, the plethora of data from the WTSI so far does not indicate a function for *Themis2* in non-immune processes with the exception of possible chromosomal instability shown through an erythrocyte micronucleus assay. Yet, no functional outcomes were observed due to chromosomal instabilities such as increased incidence of cancer in the *Themis2*$^{KO/KO}$ mouse colony or any differences in the proliferation or survival of B cells during development or after activation. However, these studies could be extended in completely *Themis2*-deficient mice using cancer models.

Since *Themis2* is also widely expressed in myeloid lineage cells, assays probing the function of these cells would be interesting as well. In the light of the immunisations performed here and in particular in terms of the influenza infection, all of which also require myeloid lineage cells, it is however questionable whether major inflammatory properties of these cells will be altered. It might be interesting to test whether *Themis2* affects

non-inflammatory functions such as wound healing, in which macrophages are involved.

For inflammatory processes it would be interesting to test the susceptibility to septic shock by injection of LPS and measuring TNFα production *in vivo* as this might lead to different results than the *in vitro* data derived from bone-marrow derived macrophages. I also did not test different adjuvants although *Themis2*$^{KO/KO}$ mice seemed to respond normally to all types of immunisation including intraperitoneal with alum, orally without adjuvant or intranasal influenza infection.

Since the only defect so far has been obtained in a Th2-driven disease, subjecting the mice to a helminth infection with pathogens such as *Heligmosomoides polygyrus* or *Nippostrongylus brasiliensis* would be an interesting set of studies to perform. Another possibility would be to test a different lung infection such as *Mycobacterium tuberculosum* if the barrier function of the epithelium is the culprit and not the fact that the disease is Th2-driven.

Lastly, since autoimmunity had not been investigated, an experimental autoimmune encephalitis experiment might give information on the function of *Themis2* particularly with regards to Breg function. I did not prioritise these experiments, as IL-10 production by B cells was normal and most autoimmunity models are strongly T cell-driven. Furthermore, this study was focussed on B cells. However, the production of autoantibodies would be an interesting area of investigation given that *Themis2* could still have a role in B cell selection similar to *Themis1* in T

cell selection. Furthermore, B cells have important roles in other autoimmune diseases that could be tested in animal models for their dependence on *Themis2* such as systemic lupus erythematosus, rheumatoid arthritis and type 1 diabetes (Hampe, 2012).

4.4 Concluding remarks

In summary, although a multitude of evidence suggested a key role for *Themis2* in B cell biology, I have shown that B cell development and activation proceed normally in its absence. In addition, I have shown that antibody responses to model antigens are normal and that resistance to influenza infection and allergy are similar in *Themis2*-deficient mice.

It remains to be determined whether *Themis2* is involved in more specialised B cell functions in a specific disease context or whether *Themis2* is involved in other biological processes in other cell types.

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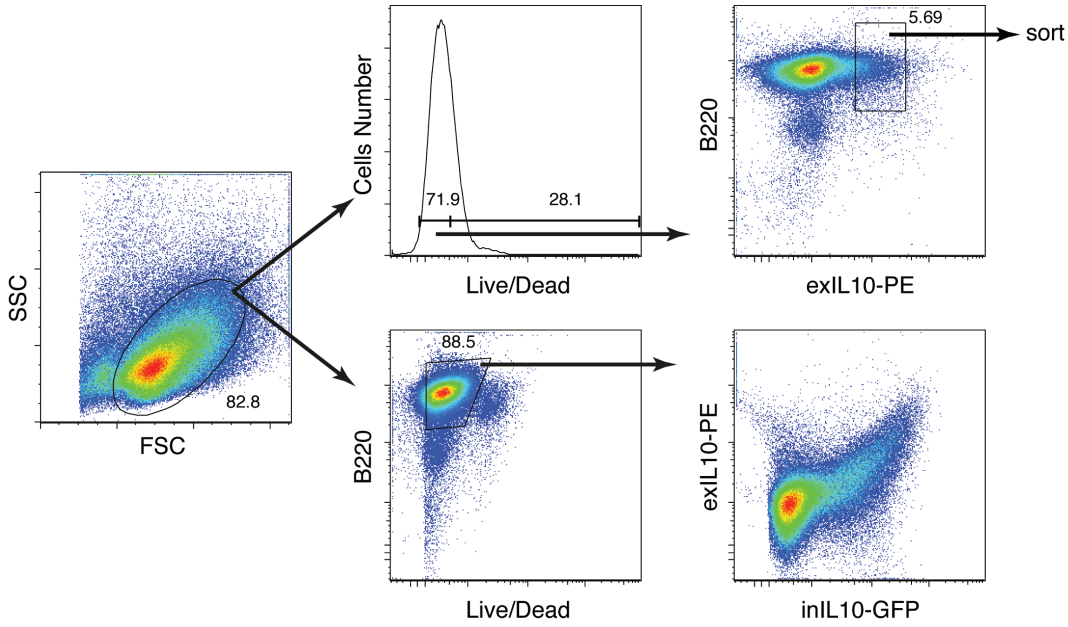
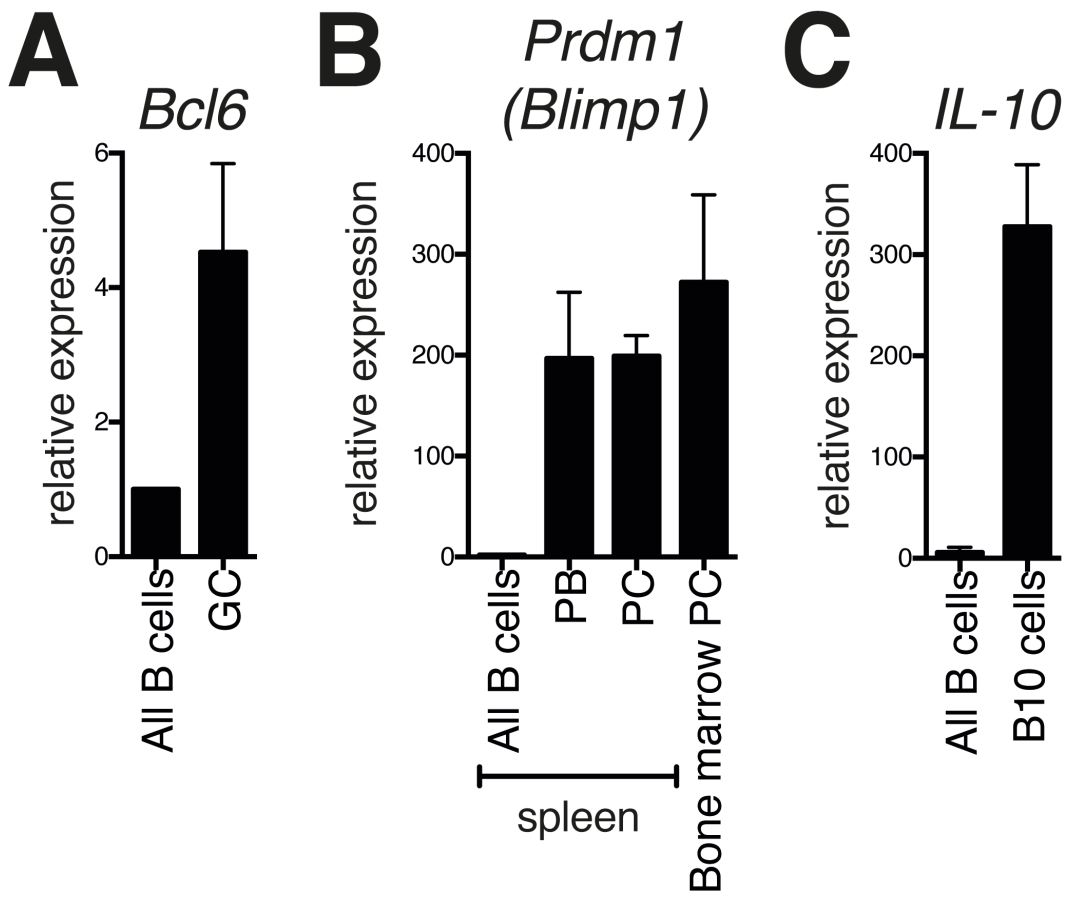
6 Appendix

6.1 Supplementary figures



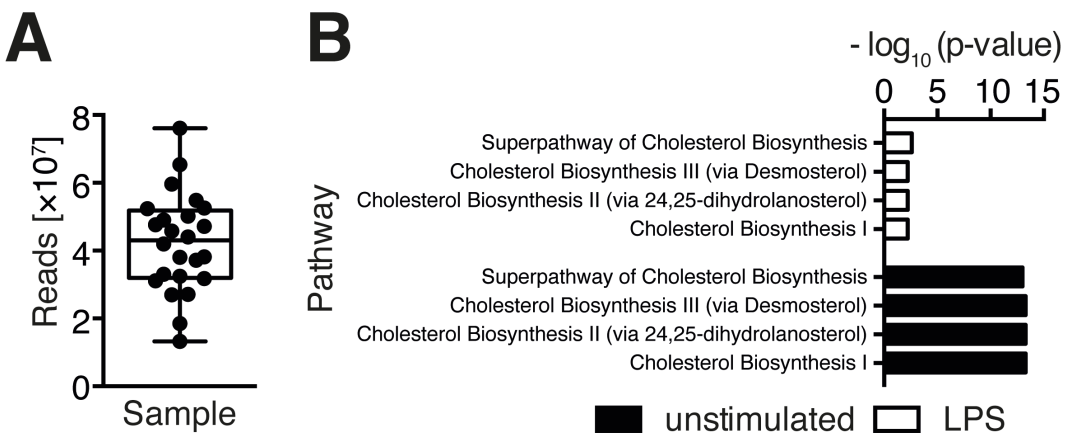
Supplementary Figure 1: Regulatory B cell sorting strategy and correlation of extracellularly captured IL-10 and IL-10 GFP reporter activity.

Regulatory B cells were sorted according to gating in the upper panel from *in vitro* cultured splenic B cells stimulated with LPS for 24 h and PMA and ionomycin for the last 5 h. The lower panel shows the correlation of secreted IL-10 that was captured on the cell surface using an anti-CD45-anti-IL-10 conjugate (exIL10-PE) and the intracellular signal from the IL-10 GFP reporter (inIL10-GFP) in control IL-10 GFP reporter tiger mouse B cells to confirm autocrine secretion and capture of IL-10 and little (false positive) paracrine capture by non-IL-10 producing-B cells.



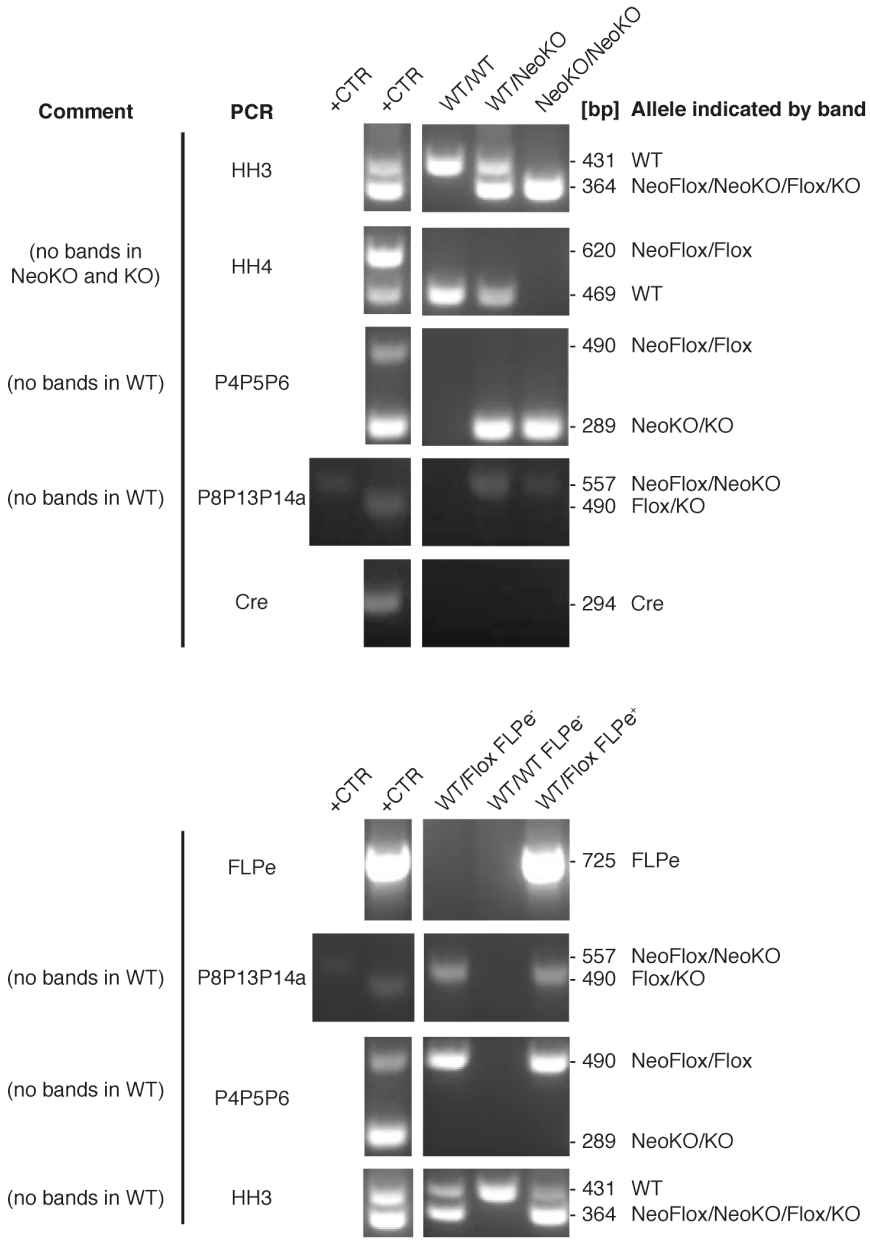
Supplementary Figure 2: Confirmation of cell identity of sorted antigen-experienced B cell subsets.

Relative expression of mRNA of lineage transcription factors or cytokines in relevant B cell populations by qRT-PCR to confirm correct sorting. **(A)** *Bcl6* mRNA is upregulated in germinal centre (GC) B cells. **(B)** *Prdm1* (*Blimp1)* is upregulated in plasmablasts (PB) and plasma cells (PC). **(C)** B10 cells express high amounts of *IL-10* mRNA. Bars show mean ± SEM of 3 biological replicates.



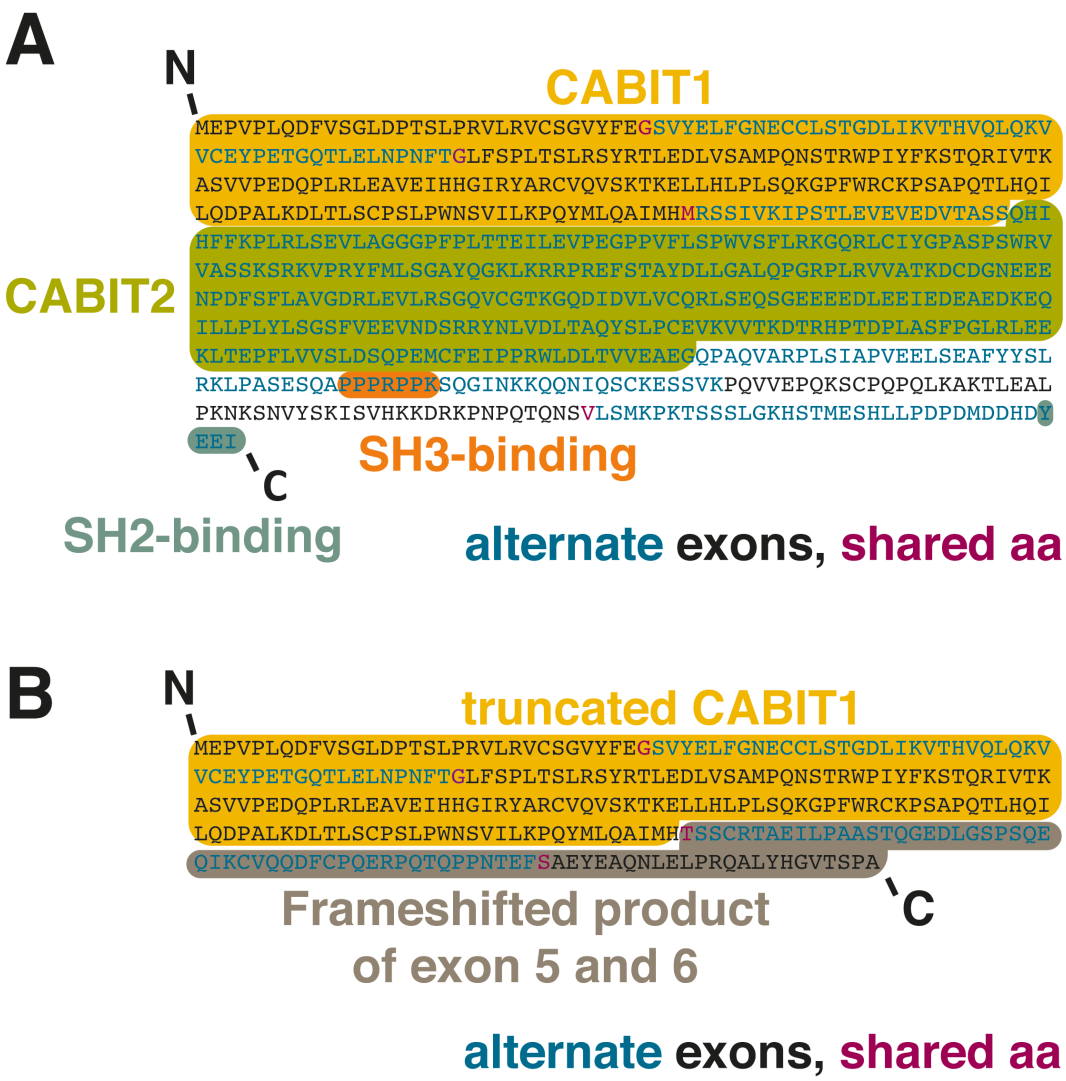
Supplementary Figure 3: RNA sequencing read depth and RNA sequencing pathway analysis of cholesterol pathways.

(A) Minimum to maximum box whisker plot of the read depth of all RNA sequencing samples in the study. All samples contained more than 1.32×10^7 reads for analysis of the transcriptome. **(B)** Statistical significance of the association of dysregulated genes between Themis2$^{KO/KO}$ and wild type follicular B cells with canonical cholesterol pathways in the pathway analyses of unstimulated or LPS-stimulated RNA sequencing samples. Low p-values indicate a high likelihood of non-random accumulation of genes from the respective metabolic pathway in the dataset of statistically significantly dysegulated genes.



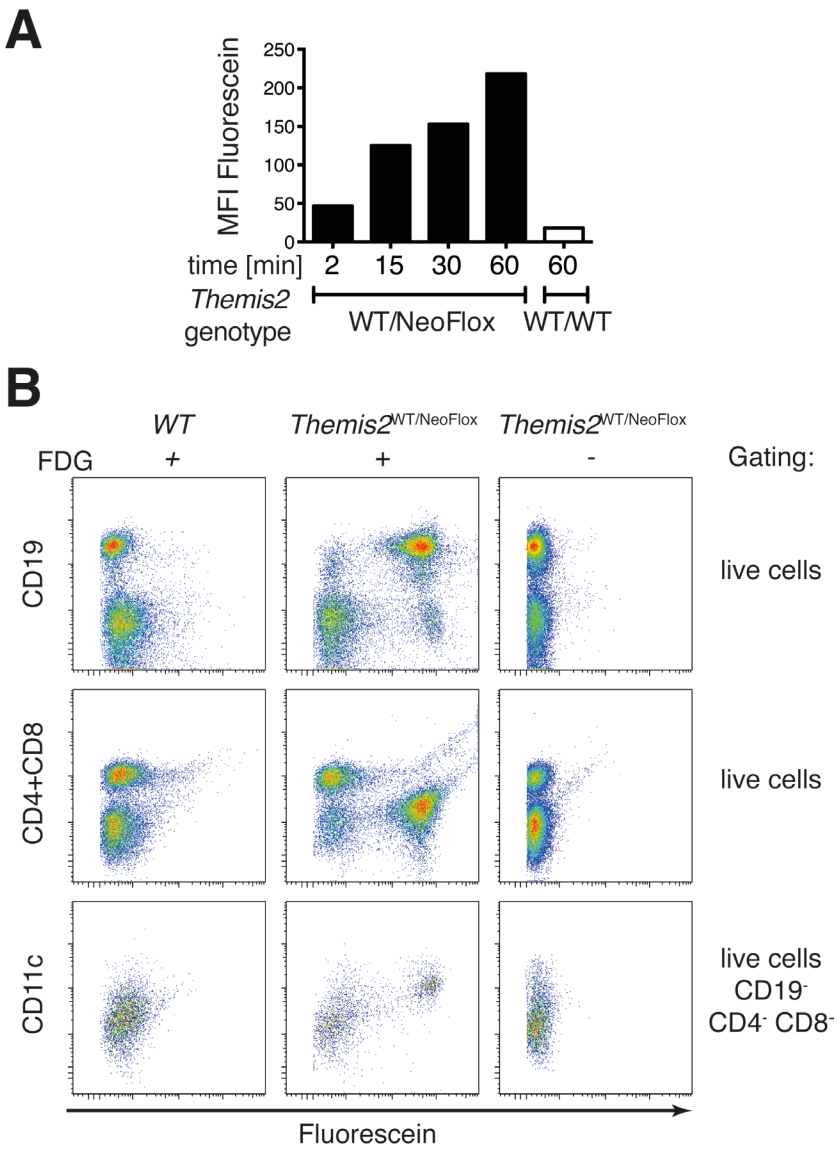
Supplementary Figure 4: Example of manual *Themis2*, *Cre* and *FLPe* genotyping.

Genotyping PCRs of a *Themis2*^{NeoKO} and a *Themis2*^{Flox} litter. PCR protocols are detailed in the section 6.2. Bands were visualised with UV light and ethidium bromide in 2 % agarose gels. Determined genotypes are labelled on top of the gel, possible genotypes due to a positive band in a particular PCR are indicated on the right. Indicated alleles describe *Themis2* alleles with the exceptions of Cre for Cre recombinase and FLPe for FLPe recombinase being present. + CTR, positive control (previously successfully amplified genomic DNA with relevant sequences present).



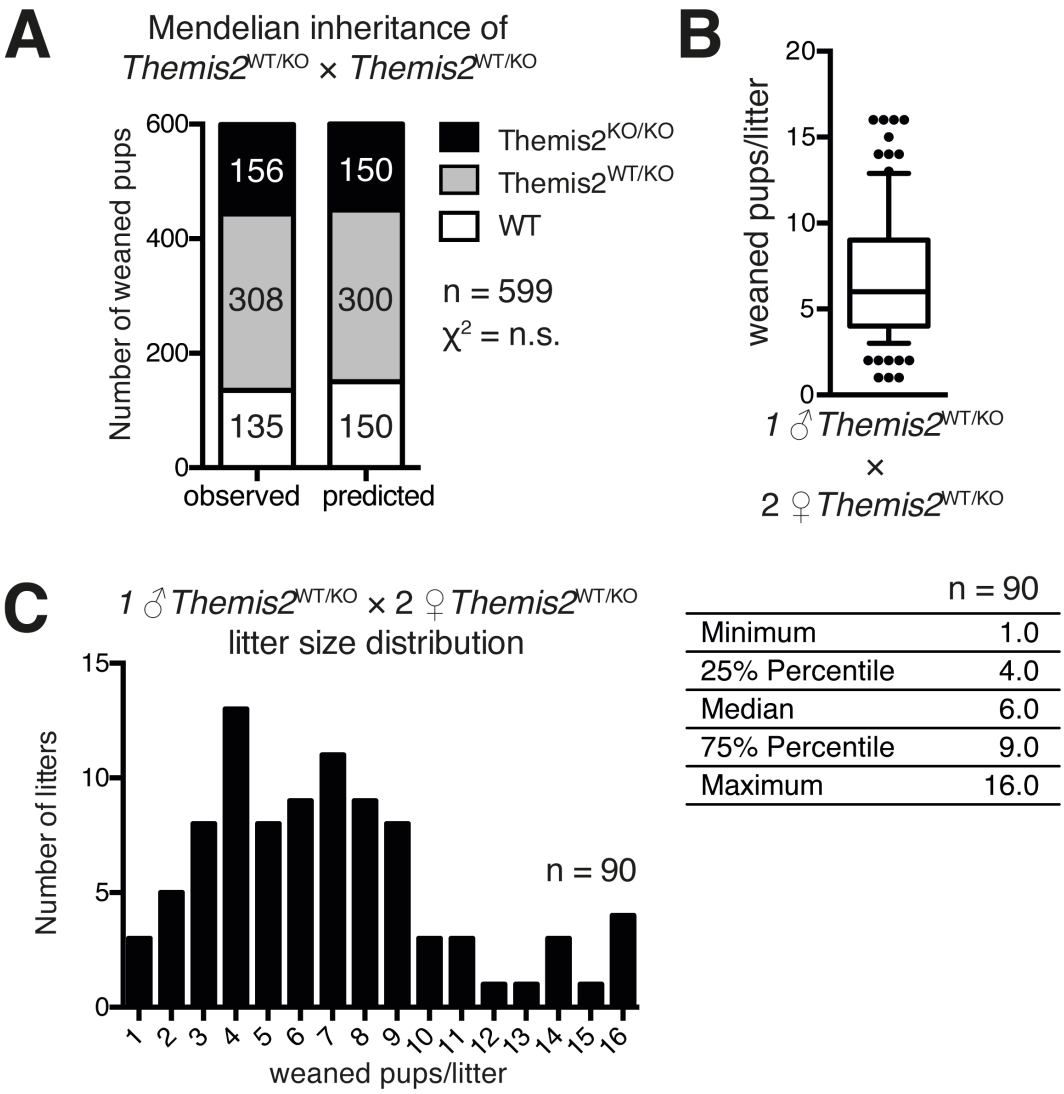
Supplementary Figure 5: Relation of *Themis2* protein sequence, exons and functional domains.

(A) Full-length murine *Themis2* in one letter amino acid code. Functional domains are overlaid and colour coded. Amino acid font colour indicates whether the encoding codon is located in an odd (black) or even (blue) numbered exon or whether the codon spans over adjacent exons (purple). **(B)** Labelled as in (A) but if exon 4 is deleted. Loss of exon 4 pushes exon 5 and 6 out of frame and produces a frameshifted C-terminus with a premature stop codon in exon 6.



Supplementary Figure 6: FACS-Gal *Themis2* promoter activity kinetics, assay validation and confirmation of cell type specificity.

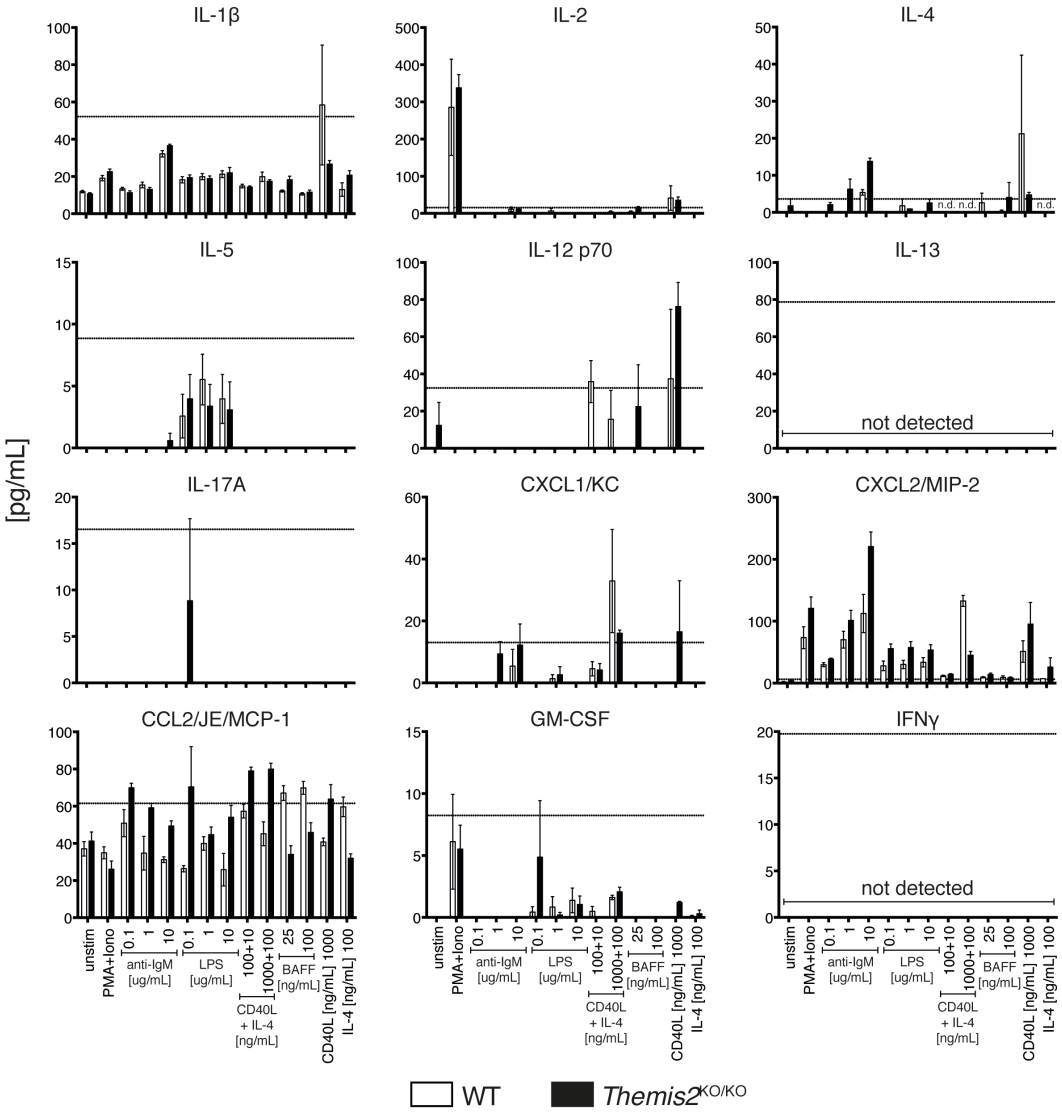
(A) Conversion of the substrate FDG into fluorescein was stopped at the indicated time points with PETG. Continual increase of the mean fluorescence intensity with longer reaction times confirm that differences in β-galactosidase expression and consequently *Themis2* promoter activity could be ideally measured at the chosen time point for analysis (45 min). **(B)** A correct *Themis2* allele (*Themis2*^{NeoFlox}) and the substrate FDG are prerequisites for a detectable fluorescein signal in splenic B cells and CD11c^{+} cells. The data further substantiate expression of *Themis2* in B cells and dendritic cells but not in T cells.



	n = 90
Minimum	1.0
25% Percentile	4.0
Median	6.0
75% Percentile	9.0
Maximum	16.0

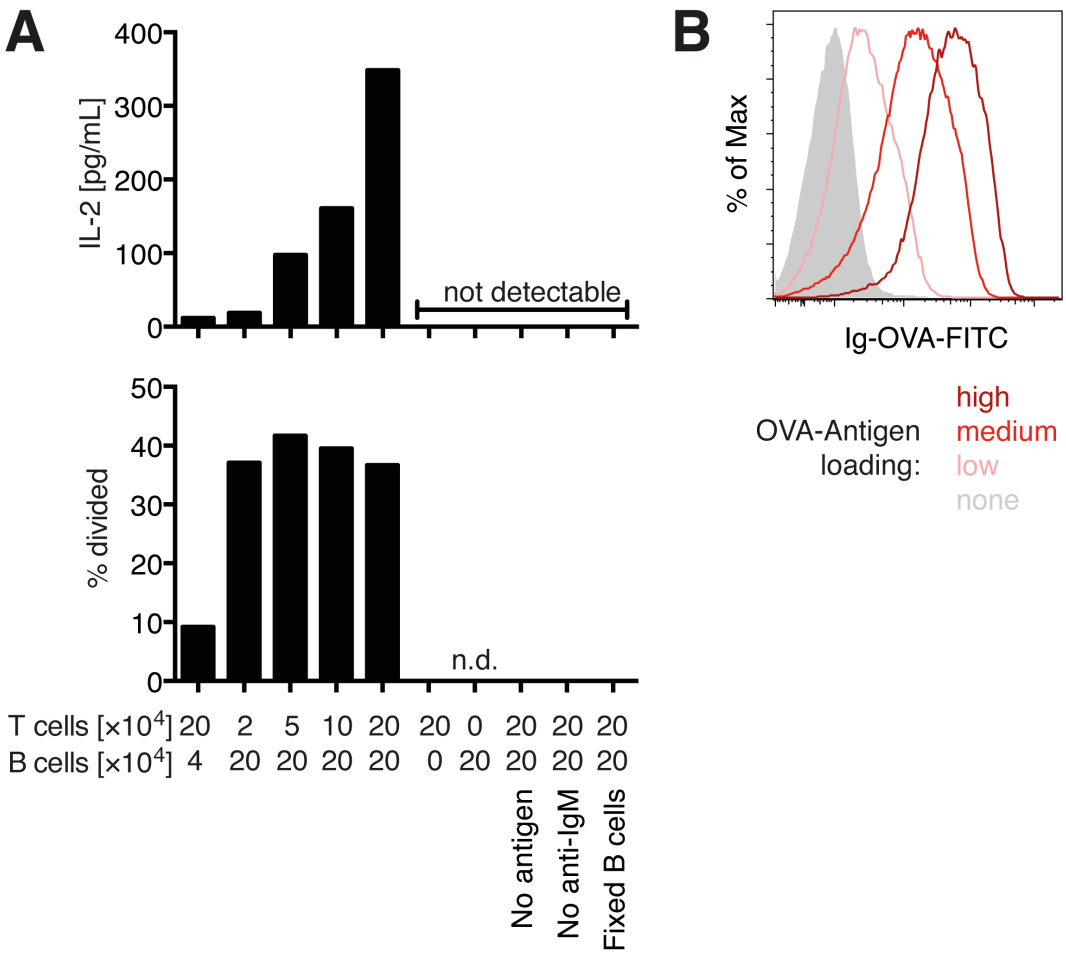
Supplementary Figure 7: *Themis2*$^{KO/KO}$ mice are produced normally from *Themis2*$^{WT/KO}$ interbreeding.

(A) *Themis2*$^{WT/KO}$ × *Themis2*$^{WT/KO}$ breeders produce expected Mendelian ratios of offspring. Statistical significance was tested using the chi-square test. n.s. not significant **(B)** 10 to 90 percentile box-whisker plot of the litter size of 1 ♂ *Themis2*$^{WT/KO}$ × 2 ♀ *Themis2*$^{WT/KO}$ mice. **(C)** Distribution plot of litter size as in (B).



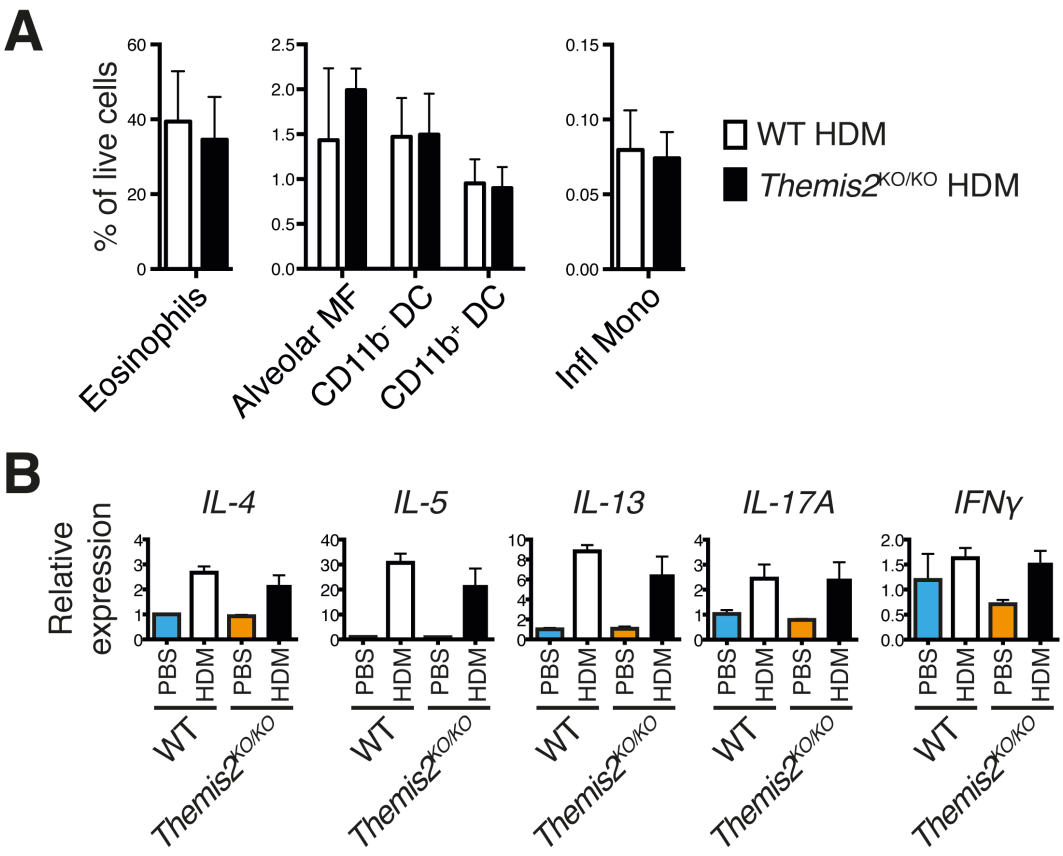
Supplementary Figure 8: *Themis2*-deficient B cells show no aberrant production of cytokines upon *in vitro* activation.

Cytokines determined by Luminex 16-plex assay in the supernatant of splenic B cells from *Themis2*$^{KO/KO}$ or wild type control animals cultured for 72 h in the presence of the indicated stimuli. Graphs show mean ± SEM of 3 biological replicates and is representative of 2 – 3 independent experiments. Dotted lines indicate reliable detection limit. n.d. not determined due to exogenously added IL-4.



Supplementary Figure 9: Assay validation of antigen-presentation assay.

(A) IL-2 production and percentage of divided OT-II T cells at different B and T cell densities or negative controls without B cells or T cells or antigen-loading or IgM cross-linking or IgM-cross-linked and antigen-loaded but paraformaldehyde fixed. Data indicated the optimal assay conditions to be 10^5 T cells co-cultured with 2×10^5 B cells as IL-2 was in an optimal range for the ELISA and around 40 % of the T cells divided. None of the negative controls resulted in any significant T cell proliferation or IL-2 production. n.d. not determined. **(B)** OVA-antigen loading on splenic B cells ($CD19^+$ $B220^+$) after titration of anti-IgM-$F(ab)_2$-biotin (high = 2.5 μg/mL, medium = 0.25 μg/mL, low = 0.025 μg/mL or nil) and secondary staining with a constant concentration of anti-biotin-Ig-OVA-FITC. Antigen-loading was measured by flow cytometry.



Supplementary Figure 10: In an acute HDM allergy model *Themis2*-deficient mice have normal expression of T helper cell cytokines in the lung and normal ratios of cell types recruited into the BAL.

(A) Percentage of various cell types among live cells in the BAL of *Themis2*-deficient or control mice on day 32 of an acute HDM allergy model. Cells were defined as in Figure 29. Bars show mean ± SEM of 4 – 5 biological replicates/group. **(B)** Relative mRNA levels of signature T helper cell cytokines in the superior lobe of the lung of *Themis2*-deficient or control mice determined by qRT-PCR. Bars show mean ± SEM of 3 (PBS) or 5 (HDM) biological replicates/group.

6.2 PCR protocols

6.2.1 Genotyping protocol HH3 for wild type and targeted *Themis2* allele

Volume/ reaction [µL]	Final concentration	Reagent
2.50	1x	10 x Reaction Buffer IV
3.50	3.5 mM	$MgCl_2$ 25mM
0.40	0.4 mM	dNTP mix 25 mM
0.50	250 nM	HH3 - 12.5 pmol/mL
0.50	250 nM	EAS_90 - 12.5 pmol/mL
0.50	250 nM	EAS_92 - 12.5 pmol/mL
0.10	1 U/mL	Thermoprimer Plus DNA Pol 250 U/mL
16.00		PCR quality H_2O
1.00	~ 4 ng/mL	Template
24		VOLUME MASTERMIX
25		TOTAL VOLUME

Step	Temp [°C]	Time	Cycles
1st Denature	94	5 min	
Denature	94	30 s	
Anneal	61	30 s	31
Extend	72	30 s	
Final extend	72	5 min	
Hold	4	forever	

Allele	Expected band size [bp]
wt	431
NeoFlox	364
Flox	364
NeoKO	364
KO	364

Primer name	5' to 3' sequence
HH3:	GCTATCAGTATTTTATGATTGGGAAA
EAS_90	GGTCTGAGCTCGCCATCAGTTC
EAS_92	CAATCAGCGTAGGGCAACTG

6.2.2 Genotyping protocol HH4 for wild type and targeted Themis2 allele

Volume/ reaction [µL]	Final concentration	Reagent
2.50	1x	10 x Reaction Buffer IV
3.50	3.5 mM	$MgCl_2$ 25mM
0.40	0.4 mM	dNTP mix 25 mM
0.50	250 nM	HH4-1 - 12.5 pmol/mL
0.50	250 nM	HH4-2 - 12.5 pmol/mL
0.50	250 nM	HH4-3 - 12.5 pmol/mL
0.10	1 U/mL	Thermoprimer Plus DNA Pol 250 U/mL
16.00		PCR quality H_2O
1.00	~ 4 ng/mL	Template
24		VOLUME MASTERMIX
25		TOTAL VOLUME

Step	Temp [°C]	Time	Cycles
1st denature	94	2 min	
Denature	94	30 s	
Anneal	61	30 s	35
Extend	72	45 s	
Final extend	72	5 min	
Hold	4	forever	

Allele	Expected band size [bp]
wt	469
NeoFlox	601 (+ 6034)
Flox	620
NeoKO	none
KO	none

Primer name	5' to 3' sequence
HH4_1	CTGAGGGATCCAACTGCTCT
HH4_2	GCCATCACGAGATTTCGATT
HH4_3	AGATCCATCGGTGACGAGAT

6.2.3 Genotyping protocol P4P5P6 for targeted Themis2 exon 4 present or deleted

Volume/ reaction [µL]	Final concentration	Reagent
2.50	1x	10 x Reaction Buffer IV
3.50	3.5 mM	$MgCl_2$ 25mM
0.40	0.4 mM	dNTP mix 25 mM
0.50	250 nM	P4 - 12.5 pmol/mL
0.50	250 nM	P5 - 12.5 pmol/mL
0.50	250 nM	P6 - 12.5 pmol/mL
0.10	1 U/mL	Thermoprimer Plus DNA Pol 250 U/mL
16.00		PCR quality H_2O
1.00	~ 4 ng/mL	Template
24		VOLUME MASTERMIX
25		TOTAL VOLUME

Step	Temp [°C]	Time	Cycles
1st Denature	94	2 min	
Denature	94	30 s	
Anneal	66	30 s	40
Extend	72	45 s	
Final extend	72	5 min	
Hold	4	forever	

Allele	Expected band size [bp]
wt	none
NeoFlox	490
Flox	490
NeoKO	289
KO	289

Primer name	5' to 3' sequence
P4	AGGAACTTCGTCGAGATAACTTCGT
P5	CCGGCACACGTCACCCAGTC
P6	CCAACGTGTCATGGCCAGCTGT

6.2.4 Genotyping protocol P8P13P14a for targeted Themis2 reporter cassette present or deleted

Volume/ reaction [µL]	Final concentration	Reagent
2.50	1x	10 x Reaction Buffer IV
3.50	3.5 mM	$MgCl_2$ 25mM
0.40	0.4 mM	dNTP mix 25 mM
0.50	250 nM	P8 - 12.5 pmol/mL
0.50	250 nM	P13 - 12.5 pmol/mL
0.50	250 nM	P14a - 12.5 pmol/mL
0.10	1 U/mL	Thermoprimer Plus DNA Pol 250U/mL
16.00		PCR quality H_2O
1.00	~ 4 ng/mL	Template
24		VOLUME MASTERMIX
25		TOTAL VOLUME

Step	Temp [°C]	Time	Cycles
1st Denature	94	5 min	
Denature	94	30 s	
Anneal	64.5	30 s	40
Extend	72	30 s	
Final extend	72	5 min	
Hold	4	forever	

Allele	Expected band size [bp]
wt	none
NeoFlox	557
Flox	490
NeoKO	557
KO	490

Primer name	5' to 3' sequence
P8	CCACAACGGGTTCTTCTGTT
P13	TGTATGCTATACGAAGTTATCTCGAC
P14a	TGCTCTGGCCTCTGTAGTCA

6.2.5 Genotyping protocol FLPe for presence of the FLPe recombinase

Volume/ reaction [µL]	Final concentration	Reagent
2.00	1x	10 x Reaction Buffer IV
1.60	2 mM	$MgCl_2$ 25mM
0.16	0.2 mM	dNTP mix 25 mM
0.80	500 nM	305 FLPe fwd Primer - 12.5 pmol/mL
0.80	500 nM	306 FLPe rev Primer - 12.5 pmol/mL
0.30	3.75 U/mL	Thermoprimer Plus DNA Pol 250U/mL
4.34		PCR quality H_2O
10.00	~ 4 ng/mL	Template
10		VOLUME MASTERMIX
20		TOTAL VOLUME

Step	Temp [°C]	Time	Cycles
1st Denature	94	3 min	
Denature	94	30 s	
Anneal	58	1 min	35
Extend	72	1 min	
Final extend	72	10 min	
Hold	4	forever	

Allele	Expected band size [bp]
wt	none
$FLPe^+$	725

Primer name	5' to 3' sequence
305 FLPe fwd	CACTGATATTGTAAGTAGTTTGC
306 FLPe rev	CTAGTGCGAAGTAGTGATCAGG

6.2.6 Genotyping protocol Cre for presence of the Cre recombinase

Volume/ reaction [µL]	Final concentration	Reagent
2.00	1x	10 x Reaction Buffer IV
1.60	2 mM	$MgCl_2$ 25mM
0.40	0.5 mM	dNTP mix 25 mM
0.63	393 nM	Cre Primer fwd - 12.5 pmol/mL
0.63	393 nM	Cre Primer rev - 12.5 pmol/mL
0.30	3.75 U/mL	Thermoprimer Plus DNA Pol 250U/mL
13.45		PCR quality H_2O
1.00	~ 4 ng/mL	Template
19		VOLUME MASTERMIX
20		TOTAL VOLUME

Step	Temp [°C]	Time	Cycles
1st Denature	94	3 min	
Denature	94	40 s	
Anneal	62	40 s	30
Extend	72	30 s	
Final extend	72	5 min	
Hold	4	forever	

Allele	Expected band size [bp]
wt	none
Cre^+	294

Primer name	5' to 3' sequence
Cre Primer fwd	TTCCCGCAGAACCTGAAGATGTTCG
Cre Primer rev	GCCAGATTACGTATATCCTGGCAGC

6.2.7 PCR protocol for presence of the *Themis2* splice variants

Volume/ reaction [µL]	Final concentration	Reagent
2.50	1x	10 x Reaction Buffer IV
3.50	3.5 mM	$MgCl_2$ 25mM
0.40	0.4 mM	dNTP mix 25 mM
0.50	250 nM	Forward Primer 12.5 pmol/uL
0.50	250 nM	Reverse Primer 12.5 pmol/uL
0.10	1 U/mL	Thermoprimer Plus DNA Pol 250U/mL
16.50		PCR quality H_2O
1.00	~ 4 ng/mL	Template
24		VOLUME MASTERMIX
25		TOTAL VOLUME

Step	Temp [°C]	Time	Cycles
1st Denature	94	2 min	
Denature	94	30 s	
Anneal	Gradient: 50-65	30 s	40
Extend	72	2 min 30 s	
Final extend	72	10 min	
Hold	4	forever	

Amplicon (Primer Pairs)	Expected band size for all exons used [bp]
P1f + P1r	1973
P2f + P2r	2080
P3f + P3r	2279
P3f + P3r2	1943

Primer name	5' to 3' sequence
P1f	GGTCTGCTCCGGAGTCTACTT
P1r	CTGGAAATGTGGCTTCCCTA
P2f	CTAGTCGGAACGCGACCATGGAG
P2r	TCCGAGTCCTGTGTGGTGGCA
P3f	GCCTGGACCCCACCTCCCTT
P3r	TGAGAGGCAGGGCGATGCTGA
P3r2	GTCATGGTCATCCATATCCGGGTCA

6.3 Differentially expressed genes between Themis2-deficient and wild type control follicular B cells

Below a list of the significantly differentially expressed genes from experiments shown in Figure 14 (unstimulated) and in Figure 20 (anti-IgM or LPS or CD40L and IL-4 stimulated). Genes are ordered by fold change and only genes with a false discovery rate of < 0.05 are reported. A more comprehensive list including normalised read counts and read densities for every gene of every sample can be found under the following link:

http://www.jimmunol.org/content/suppl/2014/06/06/jimmunol.1400943.DCSupplemental/JI_1400943_Supplemental_Material_1.xlsx

6.3.1 Legend for differentially expressed genes

Explanations of the headings for the lists below.

Ensembl gene ID	Ensembl Identifier of the gene
log_2 FC KO/WT	Logarithm to the base of 2 of the fold change of the average normalised count data in *Themis2*$^{KO/KO}$ divided by WT
FDR	False discovery rate of exact binomial test between *Themis2*$^{KO/KO}$ and WT
Gene Name	Associated gene name derived from Ensembl

6.3.2 Differentially expressed genes without stimulation

Differences in gene expression between *Themis2*$^{KO/KO}$ and WT follicular B cells without stimulation. Genes with an FDR < 0.05 are reported and ordered by fold change.

Ensembl gene ID	log_2 FC KO/WT	FDR	Gene Name
ENSMUSG00000029657	0.6255	0.006062526	Hsph1
ENSMUSG00000021250	0.5827	0.020225123	Fos
ENSMUSG00000089774	0.4532	0.032833023	Slc5a3
ENSMUSG00000037432	0.3785	0.046491211	Fer1l5
ENSMUSG00000030357	0.3605	0.000648205	Fkbp4
ENSMUSG00000076508	0.3441	0.012940005	Igkv17-127
ENSMUSG00000022403	0.3357	0.009779242	St13
ENSMUSG00000035024	0.3341	0.033281545	Ncapd3
ENSMUSG00000079003	0.3234	0.002394673	Samd1
ENSMUSG00000032122	0.3195	0.005084398	Slc37a2
ENSMUSG00000076514	0.3162	0.035814877	Igkv17-121
ENSMUSG00000020048	0.3158	0.004611779	Hsp90b1
ENSMUSG00000025316	0.2785	0.000154012	Banp
ENSMUSG00000001829	0.2590	0.036545401	Clpb
ENSMUSG00000002396	0.2514	0.045642572	Ocel1
ENSMUSG00000037933	0.2475	0.001212645	Bicd2
ENSMUSG00000003039	0.2335	0.006780471	Fam32a
ENSMUSG00000043384	0.2325	0.017089317	Gprasp1
ENSMUSG00000010755	0.2276	0.006062526	Cars
ENSMUSG00000027248	0.2269	0.030647881	Pdia3
ENSMUSG00000003814	0.2134	0.017089317	Calr
ENSMUSG00000059939	0.2097	0.032833023	9430015G10Rik
ENSMUSG00000023994	0.2028	0.046491211	Nfya
ENSMUSG00000039191	-0.2088	0.038671449	Rbpj
ENSMUSG00000031167	-0.2189	0.009667861	Rbm3
ENSMUSG00000037355	-0.2560	0.017089317	Uvssa
ENSMUSG00000022048	-0.2580	0.046491211	Dpysl2
ENSMUSG00000032018	-0.2743	0.030647881	Sc5d
ENSMUSG00000028015	-0.2744	0.000527652	Ctso
ENSMUSG00000042851	-0.3390	0.01008288	Zc3h6
ENSMUSG00000090115	-0.3448	0.034883705	Usp49
ENSMUSG00000079470	-0.3514	0.025135359	Utp14b
ENSMUSG00000021273	-0.3843	0.01337623	Fdft1
ENSMUSG00000037731	-0.4316	1.04E-10	Themis2

ENSMUSG00000031604	-0.4327	0.016188475	Sc4mol
ENSMUSG00000001467	-0.4545	0.014751047	Cyp51
ENSMUSG00000038264	-0.4584	0.044880386	Sema7a
ENSMUSG00000059743	-0.4679	0.009667861	Fdps
ENSMUSG00000032360	-0.4717	0.01337623	Hcrtr2
ENSMUSG00000045294	-0.4936	0.030647881	Insig1
ENSMUSG00000022351	-0.5504	0.042373282	Sqle
ENSMUSG00000034926	-0.6027	0.006062526	Dhcr24
ENSMUSG00000037443	-1.6311	1.24E-75	Cep85
ENSMUSG00000096979	-2.5871	5.80E-44	AL627077.2
ENSMUSG00000075014	-4.5080	6.11E-07	Gm10800

6.3.3 Differentially expressed genes after anti-IgM stimulation

Differences in gene expression between *Themis2*$^{KO/KO}$ and WT follicular B cells after 6 h stimulation with anti-IgM *in vitro*. Genes with an FDR < 0.05 are reported and ordered by fold change.

Ensembl gene ID	log_2 FC KO/WT	FDR	Gene Name
ENSMUSG00000058626	4.6780	1.11E-06	Capn11
ENSMUSG00000090186	1.0806	0.000610599	Gm7592
ENSMUSG00000062783	0.9909	0.000823014	Csprs
ENSMUSG00000014932	0.9412	3.70E-05	Yes1
ENSMUSG00000032715	0.9317	0.000552479	Trib3
ENSMUSG00000079457	0.8264	0.005467994	Gm7609
ENSMUSG00000097636	0.7652	0.014200861	AC123702.1
ENSMUSG00000038807	0.7558	0.015465071	Rap1gap2
ENSMUSG00000031327	0.7238	0.024853785	Chic1
ENSMUSG00000062157	0.6164	0.029581645	Ifnlr1
ENSMUSG00000049321	0.5690	0.04325069	Zfp2
ENSMUSG00000079190	0.5102	0.010040936	AC133103.1
ENSMUSG00000033111	0.4960	0.048051235	3830406C13Rik
ENSMUSG00000024989	0.4942	0.029819884	Cep55
ENSMUSG00000033883	0.4251	0.012906528	D3Ertd254e
ENSMUSG00000044501	0.3992	0.03989322	Zfp758
ENSMUSG00000040321	0.3965	0.006303137	Zfp770
ENSMUSG00000020305	0.3808	0.0442941	Asb3
ENSMUSG00000072568	0.3598	0.036144598	Fam84b
ENSMUSG00000022237	0.3584	0.014200861	Ankrd33b
ENSMUSG00000019699	0.3508	0.005674529	Akt3
ENSMUSG00000089788	0.3470	0.014200861	Gm16586
ENSMUSG00000035367	0.3464	0.041925775	Rmi1
ENSMUSG00000057842	0.3428	0.016162715	Zfp595
ENSMUSG00000021597	0.3390	0.018717474	Ankrd32
ENSMUSG00000090015	0.3368	0.041440983	Gm15446
ENSMUSG00000066798	0.3272	0.007299815	Zbtb6
ENSMUSG00000044763	0.3246	0.000552479	Trmt10c
ENSMUSG00000063894	0.3151	0.015855587	Zkscan8
ENSMUSG00000049164	0.3115	0.010040936	Zfp518a
ENSMUSG00000067942	0.2736	0.0442941	Zfp160
ENSMUSG00000056260	0.2699	0.041677391	Lrif1
ENSMUSG00000046785	0.2674	0.024853785	Epm2aip1
ENSMUSG00000030660	0.2599	0.033167039	Pik3c2a

ENSMUSG00000047694	0.2570	0.047746315	Yipf6
ENSMUSG00000037795	0.2394	0.03302315	N4bp2
ENSMUSG00000024943	0.2385	0.039193798	Smc5
ENSMUSG00000018287	-0.2016	0.047746315	Spag7
ENSMUSG00000036646	-0.2072	0.047746315	Man1b1
ENSMUSG00000029028	-0.2203	0.047548911	Lrrc47
ENSMUSG00000061032	-0.2224	0.041440983	Rrp1
ENSMUSG00000022556	-0.2229	0.048177636	Hsf1
ENSMUSG00000005299	-0.2230	0.047746315	Letm1
ENSMUSG00000028677	-0.2233	0.0389756	Rnf220
ENSMUSG00000024098	-0.2287	0.038814332	Twsg1
ENSMUSG00000038485	-0.2328	0.01736433	Socs7
ENSMUSG00000004929	-0.2335	0.031678855	Thop1
ENSMUSG00000020802	-0.2339	0.027578565	Ube2o
ENSMUSG00000018040	-0.2439	0.027379742	Rrp7a
ENSMUSG00000025858	-0.2502	0.034089579	Get4
ENSMUSG00000009293	-0.2528	0.041440983	Ube2g2
ENSMUSG00000018042	-0.2572	0.041925775	Cyb5r3
ENSMUSG00000020821	-0.2599	0.047746315	Kif1c
ENSMUSG00000004054	-0.2619	0.0442941	Map3k11
ENSMUSG00000029507	-0.2630	0.014200861	Pus1
ENSMUSG00000030814	-0.2652	0.019373997	Bcl7c
ENSMUSG00000020235	-0.2692	0.041925775	Fzr1
ENSMUSG00000044894	-0.2738	0.024853785	Uqcrq
ENSMUSG00000090841	-0.2759	0.027945346	Myl6
ENSMUSG00000041697	-0.2764	0.018705291	Cox6a1
ENSMUSG00000022193	-0.2793	0.041440983	Psmb5
ENSMUSG00000027613	-0.2804	0.014200861	Eif6
ENSMUSG00000020471	-0.2857	0.042370419	Pold2
ENSMUSG00000045365	-0.2874	0.027542731	Rbm15b
ENSMUSG00000013275	-0.2907	0.006992889	Slc41a1
ENSMUSG00000040669	-0.2928	0.0442941	Phc1
ENSMUSG00000048707	-0.2932	0.034350423	Tprn
ENSMUSG00000079478	-0.2954	0.010485901	Sssca1
ENSMUSG00000020307	-0.2970	0.031678855	Cdc34
ENSMUSG00000020917	-0.2982	0.013177668	Acly
ENSMUSG00000026930	-0.2991	0.038928909	Gpsm1
ENSMUSG00000035960	-0.3043	0.00459344	Apex1
ENSMUSG00000029622	-0.3053	0.03302315	Arpc1b
ENSMUSG00000024787	-0.3100	0.034089579	Snx15
ENSMUSG00000008193	-0.3101	0.041440983	Spib
ENSMUSG00000051403	-0.3102	0.042370419	Ppp1r37
ENSMUSG00000049686	-0.3119	0.047746315	Orai1
ENSMUSG00000054520	-0.3125	0.047746315	Sh3bp2
ENSMUSG00000018931	-0.3128	0.039193798	Gm16515
ENSMUSG00000031708	-0.3146	0.0442941	Tecr

ENSMUSG00000040435	-0.3164	0.005674529	Ppp1r15a
ENSMUSG00000010406	-0.3164	0.0442941	Mrpl52
ENSMUSG00000047945	-0.3193	0.042370419	Marcksl1
ENSMUSG00000042462	-0.3196	0.005674529	Dctpp1
ENSMUSG00000032583	-0.3218	0.041440983	Mon1a
ENSMUSG00000020219	-0.3218	0.008303634	Timm13
ENSMUSG00000023055	-0.3238	0.045838957	Calcoco1
ENSMUSG00000003528	-0.3256	0.037881174	Slc25a1
ENSMUSG00000020101	-0.3266	0.03302315	4632428N05Rik
ENSMUSG00000031158	-0.3301	0.006589499	Timm17b
ENSMUSG00000042303	-0.3322	0.011724046	Sgsm3
ENSMUSG00000030609	-0.3369	0.005674529	Aen
ENSMUSG00000006215	-0.3371	0.010485901	Zbtb17
ENSMUSG00000019433	-0.3401	0.033167039	Gipc1
ENSMUSG00000033307	-0.3406	0.03302315	Mif
ENSMUSG00000018334	-0.3475	0.000567448	Ksr1
ENSMUSG00000002820	-0.3518	0.041440983	Atg4d
ENSMUSG00000043445	-0.3563	0.041440983	Pgp
ENSMUSG00000039747	-0.3592	0.038767958	Orai2
ENSMUSG00000027952	-0.3670	0.017115305	Pmvk
ENSMUSG00000029401	-0.3682	0.031482084	Rilpl2
ENSMUSG00000024772	-0.3714	0.005363223	Ehd1
ENSMUSG00000002486	-0.3729	0.019244991	Tchp
ENSMUSG00000045349	-0.3756	0.047746315	Sh2d5
ENSMUSG00000073411	-0.3767	0.033167039	H2-D1
ENSMUSG00000029545	-0.3770	0.034089579	Acads
ENSMUSG00000061232	-0.3873	0.045067678	H2-K1
ENSMUSG00000064120	-0.3893	0.041925775	Mocs1
ENSMUSG00000037995	-0.3992	0.041440983	Igsf9
ENSMUSG00000019158	-0.4087	0.041440983	Tmem160
ENSMUSG00000003873	-0.4235	0.000755315	Bax
ENSMUSG00000040857	-0.4381	0.000610599	Erf
ENSMUSG00000030956	-0.4414	0.006303137	Fam53b
ENSMUSG00000001473	-0.4563	0.047746315	Tubb6
ENSMUSG00000033721	-0.4811	0.027578565	Vav3
ENSMUSG00000002083	-0.4823	0.027578565	Bbc3
ENSMUSG00000048895	-0.4829	0.047746315	Cdk5r1
ENSMUSG00000036278	-0.5117	0.0442941	Macrod1
ENSMUSG00000027533	-0.5366	0.010485901	Fabp5
ENSMUSG00000032369	-0.5468	0.010485901	Plscr1
ENSMUSG00000046598	-0.5881	0.047746315	Bdh1
ENSMUSG00000049892	-0.6446	0.014200861	Rasd1
ENSMUSG00000036825	-0.6500	0.045838957	Ssx2ip
ENSMUSG00000031239	-0.6847	0.006611202	Itm2a
ENSMUSG00000026482	-0.7002	0.010040936	Rgl1
ENSMUSG00000045136	-0.7247	0.000121648	Tubb2b

ENSMUSG00000059970	-0.7345	0.03302315	Hspa2
ENSMUSG00000047604	-0.7423	0.032077483	Frat2
ENSMUSG00000017639	-0.8020	0.039193798	Rab11fip4
ENSMUSG00000005125	-0.8233	0.010040936	Ndrg1
ENSMUSG00000019970	-0.8500	1.46E-09	Sgk1
ENSMUSG00000066026	-0.8530	0.011724046	Dhrs3
ENSMUSG00000014599	-0.8580	0.042370419	Csf1
ENSMUSG00000045763	-0.9028	0.024853785	Basp1
ENSMUSG00000051339	-1.1212	0.000121648	2900026A02Rik

6.3.4 Differentially expressed genes after LPS stimulation

Differences in gene expression between *Themis2*$^{KO/KO}$ and WT follicular B cells after 6 h stimulation with LPS *in vitro*. Genes with an FDR < 0.05 are reported and ordered by fold change.

Ensembl gene ID	log_2 FC KO/WT	FDR	Gene Name
ENSMUSG00000058626	2.480263396	0.004777414	Capn11
ENSMUSG00000044037	1.973208515	0.024430669	Als2cl
ENSMUSG00000031618	1.349804921	0.017005879	Nr3c2
ENSMUSG00000055809	1.113234493	0.002928018	Dnaaf3
ENSMUSG00000030742	1.065973111	0.005655804	Lat
ENSMUSG00000027978	0.975698515	0.000528639	Prss12
ENSMUSG00000062783	0.887777413	1.27E-07	Csprs
ENSMUSG00000043833	0.87712003	0.032052304	2900005J15Rik
ENSMUSG00000052512	0.820252762	0.047443908	Nav2
ENSMUSG00000045672	0.696510626	0.032052304	Col27a1
ENSMUSG00000022231	0.686339684	0.006443475	Sema5a
ENSMUSG00000014686	0.654869288	0.024430669	Ceacam16
ENSMUSG00000014932	0.600435236	0.006879221	Yes1
ENSMUSG00000038807	0.581313481	0.017005879	Rap1gap2
ENSMUSG00000074794	0.568167207	0.017196824	Arrdc3
ENSMUSG00000034171	0.565870251	0.007668009	Faah
ENSMUSG00000026604	0.534318554	0.012834219	Ptpn14
ENSMUSG00000079457	0.530465921	0.00600257	Gm7609
ENSMUSG00000019737	0.52457171	0.000769316	Syne4
ENSMUSG00000021094	0.5146233	0.017005879	Dhrs7
ENSMUSG00000018800	0.501527723	0.049212401	Abca5
ENSMUSG00000048581	0.482833365	0.017196824	E130311K13Rik
ENSMUSG00000040415	0.482640585	0.015501959	Dtx3
ENSMUSG00000042500	0.469092266	0.011244146	Ago4
ENSMUSG00000005686	0.449044785	0.004777414	Ampd3
ENSMUSG00000090086	0.431394289	1.60E-05	AI480526
ENSMUSG00000033209	0.40200269	0.027728504	Ttc28
ENSMUSG00000035835	0.400675822	0.001976127	BC005764
ENSMUSG00000012126	0.389477526	1.32E-05	Ubxn11
ENSMUSG00000079190	0.375132045	0.028062141	AC133103.1
ENSMUSG00000056185	0.370008259	0.032052304	Snx32
ENSMUSG00000073434	0.368052191	0.00328668	Wdr90
ENSMUSG00000032657	0.363420439	0.032052304	Fam189b
ENSMUSG00000041995	0.361868573	0.017760974	Zbed3

ENSMUSG00000097033	0.357935632	0.001330755	AC122371.1
ENSMUSG00000063897	0.347315664	0.000570831	CAAA01118383.1
ENSMUSG00000053560	0.345686402	0.012782187	Ier2
ENSMUSG00000024462	0.344710854	0.002747661	Gabbr1
ENSMUSG00000012123	0.339863288	0.032052304	Aim1l
ENSMUSG00000015335	0.338145559	0.02120223	Zdhhc12
ENSMUSG00000034833	0.330323277	0.017005879	Tespa1
ENSMUSG00000024330	0.324833332	0.031712303	Col11a2
ENSMUSG00000000682	0.316593375	0.017196824	Cd52
ENSMUSG00000072653	0.304456376	0.027675265	Zfp783
ENSMUSG00000034121	0.299329681	0.013063051	Mks1
ENSMUSG00000067370	0.299017907	0.040973344	B3galt4
ENSMUSG00000052837	0.295864756	0.039131322	Junb
ENSMUSG00000022372	0.294199349	0.009327152	Sla
ENSMUSG00000029860	0.289112225	0.015501959	Zyx
ENSMUSG00000027368	0.283669666	0.007362126	Dusp2
ENSMUSG00000035545	0.280001624	0.020594716	Leng8
ENSMUSG00000095041	0.278031225	0.029143322	AC149090.1
ENSMUSG00000002763	0.276607821	0.001330755	Pex6
ENSMUSG00000037266	0.275131038	0.002658048	D4Wsu53e
ENSMUSG00000021263	0.272521332	0.020509347	Degs2
ENSMUSG00000045252	0.2722587	0.018779249	Zfp574
ENSMUSG00000037280	0.270046749	0.02611588	Galnt6
ENSMUSG00000020893	0.263739762	0.007889941	Per1
ENSMUSG00000028454	0.263367319	2.77E-05	Pigo
ENSMUSG00000024869	0.261314133	0.007889941	Nudt8
ENSMUSG00000019039	0.259935897	0.011244146	Dalrd3
ENSMUSG00000023908	0.259622088	0.015968759	Pkmyt1
ENSMUSG00000025409	0.258973899	0.030110692	Mbd6
ENSMUSG00000057411	0.257131462	0.014517648	Fam173a
ENSMUSG00000030741	0.256236361	0.004100609	Spns1
ENSMUSG00000002668	0.254487947	0.009327152	Dennd1c
ENSMUSG00000057948	0.253762738	0.007362126	Unc13d
ENSMUSG00000071649	0.252776235	0.017410547	B3gat3
ENSMUSG00000020894	0.251407776	0.000769214	Vamp2
ENSMUSG00000040712	0.251178247	0.024430669	Camta2
ENSMUSG00000029404	0.249972091	0.01089652	Arl6ip4
ENSMUSG00000066892	0.249778806	0.007889941	Fbxl12
ENSMUSG00000038644	0.245772056	0.035738551	Pold1
ENSMUSG00000022559	0.241503528	0.011244146	Fbxl6
ENSMUSG00000023764	0.239119096	0.00876282	Sfi1
ENSMUSG00000060862	0.23906385	0.009112702	Zbtb40
ENSMUSG00000027947	0.238831675	0.030110692	Il6ra
ENSMUSG00000025572	0.235349296	0.020594716	Tmc6
ENSMUSG00000026944	0.234084982	0.017196824	Abca2
ENSMUSG00000045193	0.228077809	0.024430669	Cirbp

ENSMUSG00000037679	0.227172522	0.002658048	Inf2
ENSMUSG00000060601	0.223560785	0.035738551	Nr1h2
ENSMUSG00000020153	0.221108927	0.043585871	Ndufs7
ENSMUSG00000054874	0.220083464	0.01170624	Pcnxl3
ENSMUSG00000024180	0.2181656	0.034315608	Tmem8
ENSMUSG00000029602	0.217878252	0.03479342	Rasal1
ENSMUSG00000039205	0.214389643	0.031712303	Ciz1
ENSMUSG00000092417	0.213614518	0.039804124	Gpank1
ENSMUSG00000039263	0.211123709	0.017410547	Npepl1
ENSMUSG00000041354	0.209376119	0.045055342	Rgl2
ENSMUSG00000030337	0.200996493	0.017005879	Vamp1
ENSMUSG00000030871	0.197417886	0.035738551	Ears2
ENSMUSG00000026213	0.197057581	0.032052304	Stk11ip
ENSMUSG00000042404	0.19435636	0.014895777	Dennd4b
ENSMUSG00000047767	0.193377734	0.026043662	Atg16l2
ENSMUSG00000004661	0.192145023	0.017005879	Arid3b
ENSMUSG00000022946	0.185536103	0.048223905	Dopey2
ENSMUSG00000044763	0.18424977	0.017005879	Trmt10c
ENSMUSG00000015377	0.18207532	0.008785062	Dennd6b
ENSMUSG00000038342	0.181029867	0.024430669	Mlxip
ENSMUSG00000001127	0.17831433	0.021297209	Araf
ENSMUSG00000056260	0.177720204	0.040106564	Lrif1
ENSMUSG00000020455	0.171269617	0.031712303	Trim11
ENSMUSG00000026229	-0.142570584	0.039811771	Psmd1
ENSMUSG00000025130	-0.142784612	0.035151542	P4hb
ENSMUSG00000021595	-0.14372712	0.039811771	Nsun2
ENSMUSG00000001440	-0.144135042	0.035738551	Kpnb1
ENSMUSG00000028826	-0.147503973	0.043585871	Tmem57
ENSMUSG00000062203	-0.14796003	0.03759517	Gspt1
ENSMUSG00000020738	-0.150402356	0.048638497	Sumo2
ENSMUSG00000030168	-0.151977983	0.032052304	Adipor2
ENSMUSG00000025266	-0.153223673	0.039804124	Gnl3l
ENSMUSG00000027597	-0.156640704	0.035738551	Ahcy
ENSMUSG00000037197	-0.157463653	0.03759517	Rbm17
ENSMUSG00000078622	-0.157536186	0.021323753	Ccdc47
ENSMUSG00000030978	-0.157571216	0.042071831	Rrm1
ENSMUSG00000040331	-0.158108672	0.021297209	Nsmce4a
ENSMUSG00000027282	-0.158284708	0.025091055	Mtch2
ENSMUSG00000025591	-0.16005203	0.048638497	Tma16
ENSMUSG00000024359	-0.160699609	0.041030709	Hspa9
ENSMUSG00000031403	-0.161419191	0.048638497	Dkc1
ENSMUSG00000072872	-0.161892455	0.024430669	Rybp
ENSMUSG00000063229	-0.162515814	0.03545215	Ldha
ENSMUSG00000021024	-0.163316614	0.017760974	Psma6
ENSMUSG00000047388	-0.164665232	0.020110894	Atmin
ENSMUSG00000056851	-0.164728963	0.017196824	Pcbp2

ENSMUSG00000042487	-0.167059842	0.042071831	Leo1
ENSMUSG00000035297	-0.167199715	0.02961388	Cops4
ENSMUSG00000021650	-0.168552792	0.041030709	Ptcd2
ENSMUSG00000028416	-0.168564777	0.015968759	Bag1
ENSMUSG00000022024	-0.171716901	0.034220356	Sugt1
ENSMUSG00000027823	-0.172333636	0.043210105	Gmps
ENSMUSG00000017548	-0.174263702	0.048638497	Suz12
ENSMUSG00000037815	-0.17468012	0.02106471	Ctnna1
ENSMUSG00000053289	-0.174787238	0.005655804	Ddx10
ENSMUSG00000003814	-0.176491252	0.042071831	Calr
ENSMUSG00000049969	-0.177196441	0.040106564	Plekhf2
ENSMUSG00000030753	-0.17723496	0.011244146	Prkrir
ENSMUSG00000028452	-0.177465254	0.00514756	Vcp
ENSMUSG00000019777	-0.178517584	0.03545215	Hdac2
ENSMUSG00000023932	-0.178538106	0.030643332	Cdc5l
ENSMUSG00000030662	-0.181019508	0.022142011	Ipo5
ENSMUSG00000021408	-0.181618677	0.017760974	Ripk1
ENSMUSG00000001305	-0.182720018	0.021297209	Rrp15
ENSMUSG00000062070	-0.184846993	0.020594716	Pgk1
ENSMUSG00000020321	-0.185020588	0.01089652	Mdh1
ENSMUSG00000031954	-0.185306841	0.049778751	Cfdp1
ENSMUSG00000028633	-0.185806923	0.032052304	Ctps
ENSMUSG00000026377	-0.1870462	0.021297209	Mki67ip
ENSMUSG00000026037	-0.187155246	0.044136637	Orc2
ENSMUSG00000004043	-0.189568132	0.02106471	Stat5a
ENSMUSG00000022035	-0.189868949	0.032052304	Ccdc25
ENSMUSG00000032350	-0.191135722	0.025091055	Gclc
ENSMUSG00000031353	-0.191222944	0.014982131	Rbbp7
ENSMUSG00000045624	-0.191650389	0.044602314	Esf1
ENSMUSG00000052144	-0.193427816	0.032052304	Ppp4r2
ENSMUSG00000004707	-0.193847366	0.023672714	Ly9
ENSMUSG00000021012	-0.194738271	0.00328668	Zc3h14
ENSMUSG00000029447	-0.195032702	0.028398973	Cct6a
ENSMUSG00000024528	-0.196674288	0.037888048	Srfbp1
ENSMUSG00000020571	-0.199003155	0.011244146	Pdia6
ENSMUSG00000020917	-0.200466466	0.012920767	Acly
ENSMUSG00000020089	-0.203059718	0.007889941	Ppa1
ENSMUSG00000066441	-0.208497253	0.002928018	Rdh11
ENSMUSG00000027108	-0.208950718	0.017196824	Ola1
ENSMUSG00000026484	-0.208954982	0.014895777	Rnf2
ENSMUSG00000024165	-0.210480555	0.006254369	Hn1l
ENSMUSG00000032575	-0.215658873	0.000769316	Manf
ENSMUSG00000000628	-0.215871011	0.030110692	Hk2
ENSMUSG00000020525	-0.228378106	0.012834219	Ppm1d
ENSMUSG00000026617	-0.236002225	0.022766491	Bpnt1
ENSMUSG00000050471	-0.24283227	0.011244146	Fam118b

ENSMUSG00000024378	-0.246328957	0.029980226	Stard4
ENSMUSG00000020250	-0.24729677	0.002658048	Txnrd1
ENSMUSG00000019837	-0.250073782	0.011244146	Gtf3c6
ENSMUSG00000032324	-0.258931978	0.013147423	Tspan3
ENSMUSG00000023832	-0.259194641	0.01089652	Acat2
ENSMUSG00000032883	-0.276341238	0.022766491	Acsl3
ENSMUSG00000096336	-0.280995395	0.000528639	Igkv1-135
ENSMUSG00000021273	-0.299442846	0.01252682	Fdft1
ENSMUSG00000031349	-0.313835331	4.65E-05	Nsdhl
ENSMUSG00000021569	-0.33660424	0.040106564	Trip13
ENSMUSG00000041220	-0.350339405	0.002928018	Elovl6
ENSMUSG00000004500	-0.363611578	0.008106983	Zfp324
ENSMUSG00000094694	-0.387674461	0.020594716	Ighv1-9
ENSMUSG00000026383	-0.389855226	0.015850191	Epb4.1l5
ENSMUSG00000037731	-0.433942135	2.46E-09	Themis2
ENSMUSG00000027533	-0.507260373	0.017196824	Fabp5
ENSMUSG00000042331	-0.549446664	0.003970537	Specc1
ENSMUSG00000045136	-0.574668666	1.42E-05	Tubb2b
ENSMUSG00000004267	-0.602928681	0.003189527	Eno2
ENSMUSG00000095285	-0.70316218	0.032052304	Ighv5-9
ENSMUSG00000024665	-0.763488881	0.019916117	Fads2
ENSMUSG00000029223	-0.971341165	0.009112702	Uchl1
ENSMUSG00000037443	-1.421488041	5.71E-58	Cep85
ENSMUSG00000096979	-2.20614054	2.35E-32	AL627077.2
ENSMUSG00000065037	-2.327252008	0.007846716	Rn7sk

6.3.5 Differentially expressed genes after CD40L and IL-4 stimulation

Differences in gene expression between *Themis2*$^{KO/KO}$ and WT follicular B cells after 6 h stimulation with CD40L and IL-4 *in vitro*. Genes with an FDR < 0.05 are reported and ordered by fold change.

Ensembl gene ID	log_2 FC KO/WT	FDR	Gene Name
ENSMUSG00000065037	3.0710	0.000717349	Rn7sk
ENSMUSG00000093077	2.4765	0.001973834	Mir5105
ENSMUSG00000076258	2.3766	0.002779109	Gm23935
ENSMUSG00000098178	2.1814	0.007123548	RP23-81C12.3
ENSMUSG00000093098	2.1315	0.027714817	Gm22641
ENSMUSG00000075014	2.0613	0.048840878	Gm10800
ENSMUSG00000024066	2.0445	0.048825333	Xdh
ENSMUSG00000078763	1.9174	0.041420905	Slfn1
ENSMUSG00000034171	1.2651	0.033046943	Faah
ENSMUSG00000045092	1.1275	0.032615203	S1pr1
ENSMUSG00000020689	1.0400	0.001939409	Itgb3
ENSMUSG00000054892	0.8472	0.047696446	Txk
ENSMUSG00000062593	0.8284	0.010195312	Lilrb4
ENSMUSG00000089672	0.7783	0.007597344	Gp49a
ENSMUSG00000034833	0.7556	0.010110903	Tespa1
ENSMUSG00000094924	0.7403	4.02E-10	Cr2
ENSMUSG00000085175	0.7272	0.041099041	Gm11423
ENSMUSG00000074056	0.7260	5.50E-05	Gm10615
ENSMUSG00000052291	0.6947	0.008584951	5330438D12Rik
ENSMUSG00000097636	0.6915	0.011916895	AC123702.1
ENSMUSG00000039239	0.6886	0.029823776	Tgfb2
ENSMUSG00000025357	0.6617	0.043436593	Dgka
ENSMUSG00000073008	0.6332	3.34E-06	Gpr174
ENSMUSG00000062939	0.6295	0.000262569	Stat4
ENSMUSG00000030670	0.6239	0.023607846	Cyp2r1
ENSMUSG00000064696	0.6124	0.033724667	Gm24148
ENSMUSG00000059495	0.5857	0.032876075	Arhgef12
ENSMUSG00000032715	0.5694	0.021498388	Trib3
ENSMUSG00000050921	0.5522	0.023607846	P2ry10
ENSMUSG00000025573	0.5491	0.008584951	6030468B19Rik
ENSMUSG00000026616	0.5399	9.94E-09	Cr2
ENSMUSG00000031586	0.5280	0.043436593	Rbpms

ENSMUSG00000097571	0.5073	0.001037383	Jpx
ENSMUSG00000036339	0.5003	1.18E-05	Tmem260
ENSMUSG00000022901	0.4804	0.000380445	Cd86
ENSMUSG00000027347	0.4789	0.011916895	Rasgrp1
ENSMUSG00000053835	0.4761	0.022568087	H2-T24
ENSMUSG00000026080	0.4727	4.43E-05	Chst10
ENSMUSG00000079057	0.4644	0.045203526	Cyp4v3
ENSMUSG00000090086	0.4626	0.000717349	AI480526
ENSMUSG00000026723	0.4565	0.021645122	Trdmt1
ENSMUSG00000039853	0.4539	0.0392945	Trim14
ENSMUSG00000064337	0.4536	0.008584951	mt-Rnr1
ENSMUSG00000027010	0.4492	0.033046943	Slc25a12
ENSMUSG00000038520	0.4486	3.99E-05	Tbc1d17
ENSMUSG00000024677	0.4471	0.023607846	Ms4a6b
ENSMUSG00000024592	0.4442	0.003366903	C330018D20Rik
ENSMUSG00000060301	0.4439	0.021498388	2610008E11Rik
ENSMUSG00000030671	0.4417	0.030145051	Pde3b
ENSMUSG00000090958	0.4341	0.021645122	Lrrc32
ENSMUSG00000097347	0.4340	0.043382354	AC121292.1
ENSMUSG00000097180	0.4304	0.000717349	AC117663.1
ENSMUSG00000028391	0.4304	0.048840878	Wdr31
ENSMUSG00000039942	0.4293	0.022568087	Ptger4
ENSMUSG00000049775	0.4275	0.000548101	Tmsb4x
ENSMUSG00000055044	0.4240	0.029151962	Pdlim1
ENSMUSG00000023927	0.4173	0.00271052	Satb1
ENSMUSG00000052676	0.4153	0.027767886	Zmat1
ENSMUSG00000072568	0.4075	0.048825333	Fam84b
ENSMUSG00000072653	0.4044	0.001795787	Zfp783
ENSMUSG00000027950	0.4042	0.023748824	Chrnb2
ENSMUSG00000086859	0.3940	0.021498388	2810008D09Rik
ENSMUSG00000097061	0.3856	0.017582085	AC099934.1
ENSMUSG00000032344	0.3820	0.023960573	Mb21d1
ENSMUSG00000074785	0.3793	0.001157564	Plxnc1
ENSMUSG00000031627	0.3792	0.008173191	Irf2
ENSMUSG00000043991	0.3761	0.000295643	Pura
ENSMUSG00000026222	0.3750	0.011916895	Sp100
ENSMUSG00000054237	0.3731	0.001157564	Fra10ac1
ENSMUSG00000049950	0.3725	0.000717349	Rpp38
ENSMUSG00000039783	0.3715	0.020805166	Kmo
ENSMUSG00000085334	0.3598	0.025591231	Gm12940
ENSMUSG00000014932	0.3584	0.033046943	Yes1
ENSMUSG00000089788	0.3545	0.000140384	Gm16586
ENSMUSG00000050555	0.3522	0.007823148	Hyls1
ENSMUSG00000074733	0.3518	0.001795787	5830428H23Rik
ENSMUSG00000015790	0.3444	0.024699511	Surf1
ENSMUSG00000047242	0.3383	0.048825333	Taf9b

ENSMUSG00000024675	0.3381	0.005117236	Ms4a4c
ENSMUSG00000030031	0.3370	0.00016545	Kbtbd8
ENSMUSG00000000682	0.3334	0.032615203	Cd52
ENSMUSG00000020142	0.3333	0.002664257	Slc1a4
ENSMUSG00000074024	0.3271	0.018316719	4632427E13Rik
ENSMUSG00000095123	0.3266	0.043382354	Gm21781
ENSMUSG00000000617	0.3250	0.033770716	Grm6
ENSMUSG00000027341	0.3210	0.000295643	Tmem230
ENSMUSG00000031134	0.3164	0.000741568	Rbmx
ENSMUSG00000052137	0.3159	0.015250681	Rbm12b2
ENSMUSG00000037172	0.3152	0.032615203	E330009J07Rik
ENSMUSG00000042408	0.3089	0.019455285	Zmym6
ENSMUSG00000031246	0.3086	0.045027051	Sh3bgrl
ENSMUSG00000003038	0.3039	3.86E-05	Hmgn2
ENSMUSG00000037266	0.3007	0.002290634	D4Wsu53e
ENSMUSG00000056145	0.2962	0.036901267	AI504432
ENSMUSG00000054520	0.2925	0.022568087	Sh3bp2
ENSMUSG00000063894	0.2904	0.002734759	Zkscan8
ENSMUSG00000040747	0.2860	0.00336583	Cd53
ENSMUSG00000020894	0.2848	0.000717349	Vamp2
ENSMUSG00000026754	0.2840	0.009082003	Golga1
ENSMUSG00000047880	0.2836	0.001037383	Cxcr5
ENSMUSG00000025903	0.2827	0.023811964	Lypla1
ENSMUSG00000043090	0.2790	5.08E-05	Zfp866
ENSMUSG00000097729	0.2789	0.040452282	AC108401.2
ENSMUSG00000041235	0.2756	0.015508466	Chd7
ENSMUSG00000097195	0.2736	0.000741568	Snhg5
ENSMUSG00000040321	0.2731	0.021803315	Zfp770
ENSMUSG00000021423	0.2709	0.020275509	Ly86
ENSMUSG00000057842	0.2699	0.029823776	Zfp595
ENSMUSG00000029106	0.2688	0.040452282	Add1
ENSMUSG00000018654	0.2686	0.005446901	Ikzf1
ENSMUSG00000020069	0.2681	0.020420277	Hnrnph3
ENSMUSG00000051285	0.2677	0.00812726	Pcmtd1
ENSMUSG00000067942	0.2593	0.010124387	Zfp160
ENSMUSG00000041920	0.2583	0.023811964	Slc16a6
ENSMUSG00000034163	0.2533	0.016916868	Zfc3h1
ENSMUSG00000026767	0.2526	0.044257644	Fam188a
ENSMUSG00000040818	0.2504	0.019052569	Dennd6a
ENSMUSG00000022587	0.2472	0.041369813	Ly6e
ENSMUSG00000079659	0.2464	0.010575976	Tmem243
ENSMUSG00000030279	0.2458	0.044000261	C2cd5
ENSMUSG00000064289	0.2437	0.00314612	Tank
ENSMUSG00000026771	0.2431	0.036901267	Spopl
ENSMUSG00000046351	0.2355	0.043247235	Zfp322a
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ENSMUSG00000026600	0.2342	0.00359028	Soat1
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ENSMUSG00000026107	0.2299	0.032007878	Nabp1
ENSMUSG00000020137	0.2251	0.032876075	Thap2
ENSMUSG00000021327	0.2242	0.01467379	Zkscan3
ENSMUSG00000045140	0.2217	0.049898085	Pigw
ENSMUSG00000074578	0.2211	0.024699511	1500012F01Rik
ENSMUSG00000021510	0.2203	0.047750135	A530054K11Rik
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ENSMUSG00000092558	0.2111	0.021803315	Med20
ENSMUSG00000027763	0.2094	0.002187523	Mbnl1
ENSMUSG00000097772	0.2092	0.01551619	AC107744.1
ENSMUSG00000049164	0.2090	0.033724667	Zfp518a
ENSMUSG00000026201	0.2089	0.033466262	Stk16
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ENSMUSG00000044763	0.2052	0.006305874	Trmt10c
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ENSMUSG00000027829	0.1961	0.032991082	Ccnl1
ENSMUSG00000046785	0.1954	0.033515508	Epm2aip1
ENSMUSG00000053332	0.1889	0.049898085	Gas5
ENSMUSG00000049606	0.1871	0.047750135	Zfp644
ENSMUSG00000032244	0.1849	0.014186946	Fem1b
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ENSMUSG00000038485	-0.1767	0.01964152	Socs7
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ENSMUSG00000028334	-0.2065	0.027767886	Nans
ENSMUSG00000020781	-0.2117	0.003687863	Tsen54
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ENSMUSG00000025178	-0.2310	0.018260064	Pi4k2a
ENSMUSG00000028962	-0.2340	0.003343483	Slc4a2
ENSMUSG00000053730	-0.2357	0.033724667	Tmem39b
ENSMUSG00000034341	-0.2375	0.014221093	Wbp2
ENSMUSG00000034412	-0.2376	0.022568087	Tbc1d10a
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ENSMUSG00000024277	-0.2391	0.049276448	Mapre2
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ENSMUSG00000055067	-0.2591	0.019398411	Smyd3
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ENSMUSG00000020593	-0.2640	0.015250681	Lpin1
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ENSMUSG00000014453	-0.2828	0.003366903	Blk
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ENSMUSG00000063268	-0.2916	0.023748824	Parp10
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ENSMUSG00000028849	-0.3034	0.023607846	Map7d1
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ENSMUSG00000072825	-0.3131	0.022522148	Cep170b
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ENSMUSG00000037731	-0.3342	0.039254098	Themis2
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ENSMUSG00000019947	-0.3591	0.010908469	Arid5b
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ENSMUSG00000025533	-0.3706	0.019513011	Asl
ENSMUSG00000001473	-0.3740	0.014186946	Tubb6
ENSMUSG00000028977	-0.3759	0.027057716	Casz1
ENSMUSG00000026603	-0.3771	0.043382354	Smyd2
ENSMUSG00000018334	-0.3850	0.00271052	Ksr1
ENSMUSG00000030956	-0.3889	0.014186946	Fam53b
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ENSMUSG00000024353	-0.3992	0.043382354	Mzb1
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ENSMUSG00000031779	-0.4260	0.002059319	Ccl22
ENSMUSG00000074480	-0.4280	0.00839649	Mex3a
ENSMUSG00000039747	-0.4293	4.43E-05	Orai2
ENSMUSG00000072082	-0.4468	0.000717349	Ccnf
ENSMUSG00000032501	-0.4563	0.049898085	Trib1
ENSMUSG00000094546	-0.4747	0.010575976	Ighv1-26
ENSMUSG00000037692	-0.4864	0.014418243	Ahdc1
ENSMUSG00000047875	-0.5241	0.001238565	Gpr157
ENSMUSG00000023055	-0.5779	0.000717349	Calcoco1
ENSMUSG00000035547	-0.5855	0.044013107	Capn5
ENSMUSG00000018428	-0.5879	0.000380445	Akap1
ENSMUSG00000058173	-0.6022	0.039692064	Smco4
ENSMUSG00000038545	-0.6117	0.015250681	Cul7
ENSMUSG00000037443	-0.6231	3.00E-07	Cep85
ENSMUSG00000024665	-0.6340	0.004694979	Fads2
ENSMUSG00000006445	-0.6604	0.00812726	Epha2
ENSMUSG00000004267	-0.6830	0.010575976	Eno2
ENSMUSG00000005107	-0.6834	0.036222761	Slc2a9
ENSMUSG00000045136	-0.6954	2.21E-06	Tubb2b
ENSMUSG00000002602	-0.7300	0.000717349	Axl
ENSMUSG00000028164	-0.7625	0.000446559	Manba
ENSMUSG00000051790	-0.8183	0.026135515	Nlgn2
ENSMUSG00000032014	-0.8428	0.023895363	Oaf
ENSMUSG00000090628	-0.8586	0.032751337	Gm17083
ENSMUSG00000026223	-0.8897	0.021333128	Itm2c
ENSMUSG00000094694	-1.0240	0.003747648	Ighv1-9
ENSMUSG00000000982	-1.2176	0.048840878	Ccl3
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ENSMUSG00000096979	-1.4101	6.03E-09	AL627077.2
ENSMUSG00000051339	-1.7172	0.048260718	2900026A02Rik
ENSMUSG00000022469	-2.1600	0.04464277	Rapgef3